

Thesis for the degree of Cand. Pharm.

STUDIES AROUND THE SYNTHESIS OF  
ALBUMIN BINDING DERIVATIVES  
OF OSELTAMIVIR CARBOXYLATE



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### 3 ABBREVIATIONS

ACN	Acetonitrile
Boc <sub>2</sub> O	Di-tert-butyl dicarbonate
d	Doublet
dd	Double doublet
ddd	Double double doublet
ddt	Double double triplet
dt	double triplet
DCC	N,N-dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAP	Dimethylaminopyridine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EDCI	N-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EPR	Enhanced Permeability and Retention
FDA	Food and Drug Administration
GABA	$\gamma$ -Amino Butyric Acid
HA	Hemagglutinin
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
Hz	Hertz
m	Multiplet
mM	Milli Molar
MeOH	Methanol

NA	Neuraminidase
NMR	Nuclear Magnetic Resonance
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
OTV	Oseltamivir
OTVC	Oseltamivir carboxylate
OTVP	Oseltamivir phosphate
q	quartet
RP-18	Reverse Phase silica with 18 carbon chain
RNA	Ribonucleic acid
s	Singlet
t	Triplet
TCE	Trichloroethanol
THF	Tetrahydrofurane
tt	Triple triplet
WHO	World Health Organization

## 4 SUMMARY

A series of ester derivatives, potential prodrugs, of the antiviral agent oseltamivir carboxylate (OTVC) has been attempted synthesized. The aim was to synthesize prodrugs with high affinity for proteins compared to OTVC and with a slower conversion to the carboxylate in contrast to the ethyl ester oseltamivir (OTV) which is the active substance of Tamiflu®. The approach to increase the binding was to esterify the active compound of (OTV), OTVC, with a ligand that carry a free carboxylic acid. Such a component will carry a negative charge in vivo and be somewhat hydrophobic, and should therefore bind to albumin. Most likely, the prodrug with increased protein binding will give fewer side effects than the mother compound, due to the fact that it is highly bound to albumin rendering the free concentration low. It is also possible that a passive targeting effect can be achieved. This is because there has been observed enhanced protein retention effect in infected tissue.

Both the acid of the ligands, and the amine group of OTVC had to be protected in this synthesis in order to avoid side reactions. The amine was first tried protected with isonicotinyl. However, this synthesis proved to be troublesome. Tert - butylcarbonate was therefore chosen instead. As a protection group for the acid, trichloroethanol (TCE) was chosen.

OTV was hydrolyzed and the amine group was protected with tert – butylcarbonyl. The acid of the ligand was protected with TCE and esterified with the acid of the protected OTVC. Due to lack of time, a successful deprotection step was not achieved.

There have not been published structures of neither OTV nor OTVC based on x-ray crystallography. It was an aim of this thesis to grow crystals of these compounds, and obtain a structure based on x-ray. There were obtained crystals both structures, however, they were too small for reliable data to be obtained.



## 5 INTRODUCTION

### 5.1 *The different types of viruses*

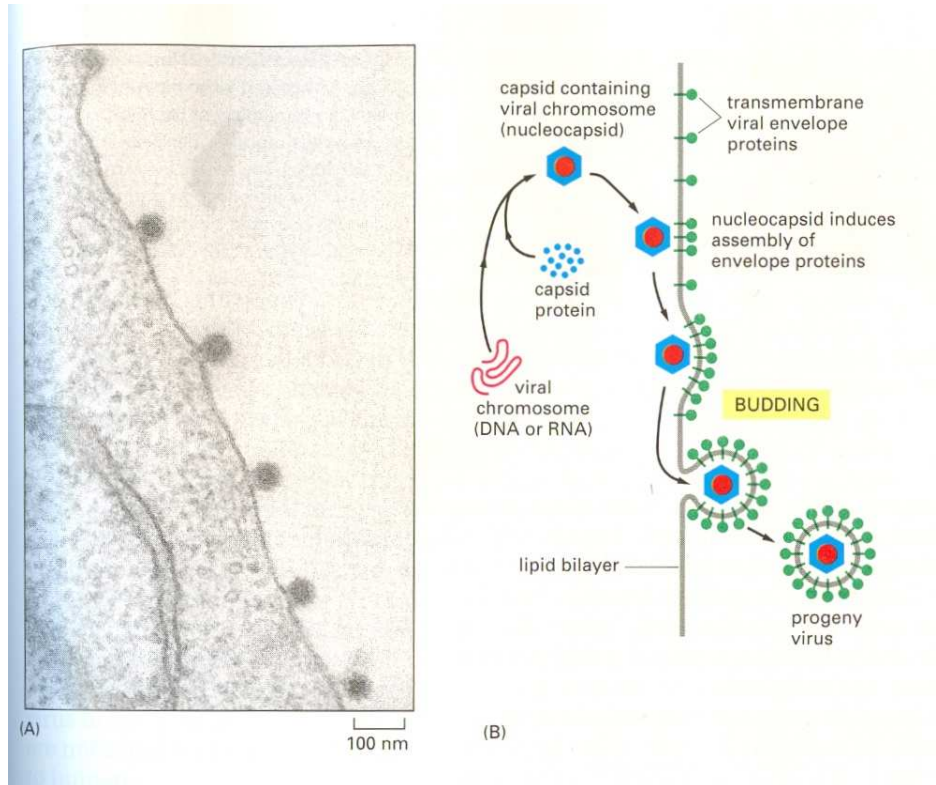
Infection by parasites is an ongoing process in all living organisms. Many of the parasites that infect humans make use of their own machinery to perform their reproduction and they rely on their host cells only for nutrients. Although they are very unpleasant guests in our body and cause death and destruction, the viruses are the ultimate parasites. They travel light and most of them carry no machinery on their own, not for replication and not for making use of any energy whatsoever. What they carry is information of how the host shall replicate them.

The viruses are not considered to be living organisms since they do not satisfy the basic criteria of life. They are acellular and do not carry out metabolism in any form; neither photosynthesis, cellular respiration, nor fermentation. At the Rockefeller Institute, Wendell Stanley was able to isolate the first virus in 1933, and discovered that viruses can best be regarded as a chemical matter, rather than life since the virus precipitated as crystals from a solution. The precipitated tobacco mosaic virus was able to infect healthy tobacco plants. This showed that the precipitate was a virus, and not just a chemical derived from it (1).

When a virus infects its selected host, it releases its genome in the cell. The genome is transcribed and translated by the cells machinery, and this produces a variety of proteins. This is the way the virus takes control over the host cell. “In general, replication involves 1) disassembly of the infectious virus particle, 2) replication of the viral genome, 3) synthesis of the viral proteins by the host cell translation machinery, and 4) reassembly of these components into progeny virus particles” (2)

All of the genomes of viruses are covered by a protein layer, synthesized by the cells machinery, ordered by the viral genome. Some of the viruses are in addition packed in a lipid envelope that originates from the cell wall of the host. These are called enveloped viruses. The viruses that do not carry such a lipid envelope multiply in so large numbers that it finally kills the cell. The cell wall bursts (a lysis occur) and the progeny virions spread out infecting nearby cells. An example of a clinical manifestation of this lysis is the cold sores caused by herpes simplex, due to the killing of epidermal cells. The enveloped viruses, on the other

hand, do not kill the host cell in the releasing process. This type of viral release is called a budding, where the intracellular viral particle is encapsulated by a part of the outer cellular membrane, as shown in Figure 1 below.



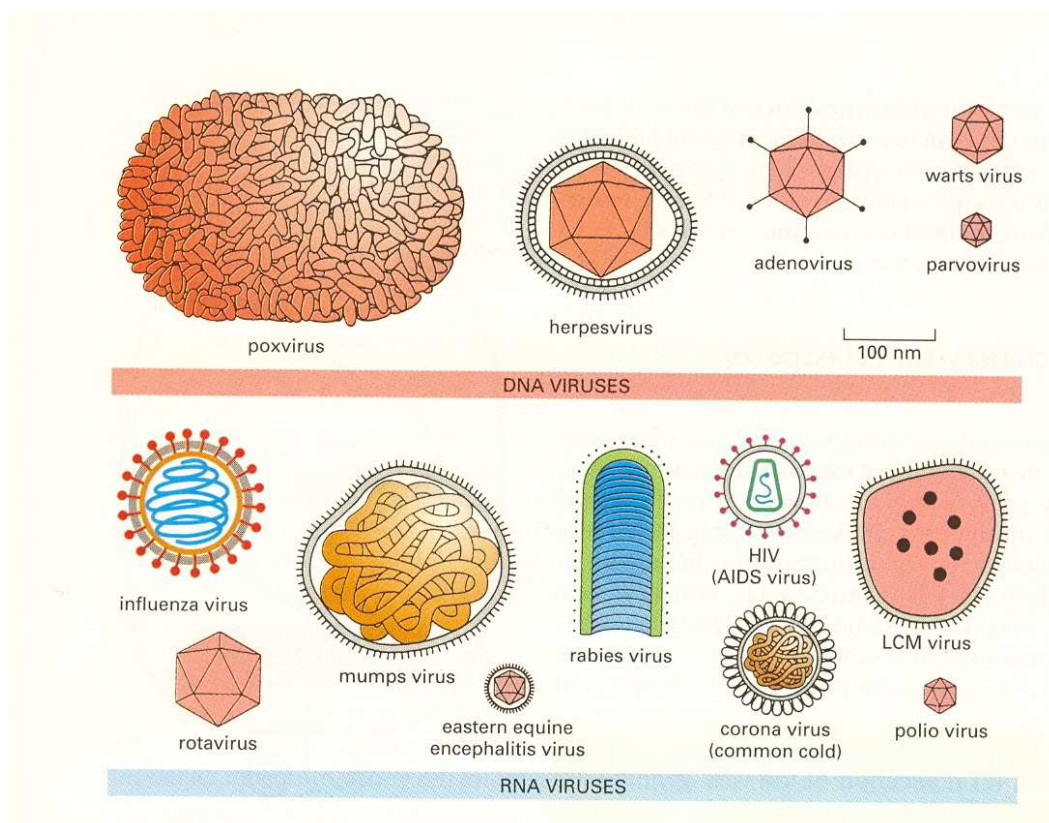
**Figure 1: (A) Electron micrograph of an animal cell from which six copies of an enveloped virus are budding. (B) Schematic view of the envelope assembly and budding process. The lipid bilayer is derived from the host cell plasma membrane. The proteins shown in green are encoded by the viral genome. Reprinted from (2).**

When the budding is complete, the progeny virus is coated with a part of the cellular membrane. As mentioned, the cell is not killed when the viruses leave the cell in a budding process, but lives on, producing new virus particles continuously. This is the reason why the enveloped viruses in general can cause chronic infections (2). The influenza virus is enveloped, however, the infection is not chronic.

There are mainly 6 families of respiratory viruses: orthomyxovirus, paramyxovirus, picornavirus, coronavirus, adenovirus and herpesvirus. Other types of viruses are listed in Table 1 on page 11.

**Table 1: An overview of some of the virus families(3, 4).**

Family	Genome	Disease
Orthomyxovirus	RNA	Influenza
Paramyxovirus	RNA	Measels and mumps
Picornavirun	RNA	Colds, meningitis, poliomyelitis
Coronavirus		
Adenovirus	DNA	Sore throat, conjunctivitis Chickenpox, shingels, cold sores and glandular
Herpesvirus	DNA	fever
Poxvirus	DNA	Smallpox
Papillomavirus	DNA	Warts
Rubella virus	RNA	German measles
Rhabdovirus	RNA	Rabies
Retrovirus	RNA	Like HIV causing AIDS
Arenavirus	RNA	meningitis
Hepandavirus	RNA	Serum hepatitis
Rabovirus	RNA	Tic borne encephalitis and yellow fever

**Figure 2: Different types of viruses. Reprinted from (2).**

## 5.2 *Viral taxonomy*

It is not clear if all the viruses have a common origin, so a Linnaean classification, which has been applied for the living organisms, is not applicable for viruses. However, a logical classification is needed to understand viruses in detail, and how to generalize them. Schemes has been worked out, classifying them regarding to their basic properties. The three most important properties are types of genome inside the capsid (DNA or RNA, single or double stranded, circular or linear, single piece or segmented), protein arrangement of the capsid (symmetry and dimensions) and other components (enveloped virus, important enzymes). This is a rough classification irrespective of what type of cell the virus infects, or what disease it causes (5). Figure 2 on page 11 show the shape and structures of different type of viruses.

### 5.2.1 The Baltimore scheme of virus classification

The Baltimore scheme of virus classification, suggested by David Baltimore in 1971, is based on the way the viruses produce mRNA. In order to have proteins synthesized, all viruses must produce mRNA in some way, but viruses differ in which genome they utilize for this. Viruses with genome of the same sense as the mRNA, are called positive (+) sense RNA viruses, while those who have the opposite sense of mRNA are called negative (-) sense RNA viruses. This classification scheme has limitations and is used mostly for classifying RNA viruses. (5) In a classification scheme based on the Baltimore classification and the three basic properties mentioned above, the influenza virus is classified like this: Single stranded RNA virus – Negative sense RNA – Envelope – Helical – Segmented genome. In this class we find the orthomyxovirus, causing influenza.

## 5.3 *Influenza and avian flu*

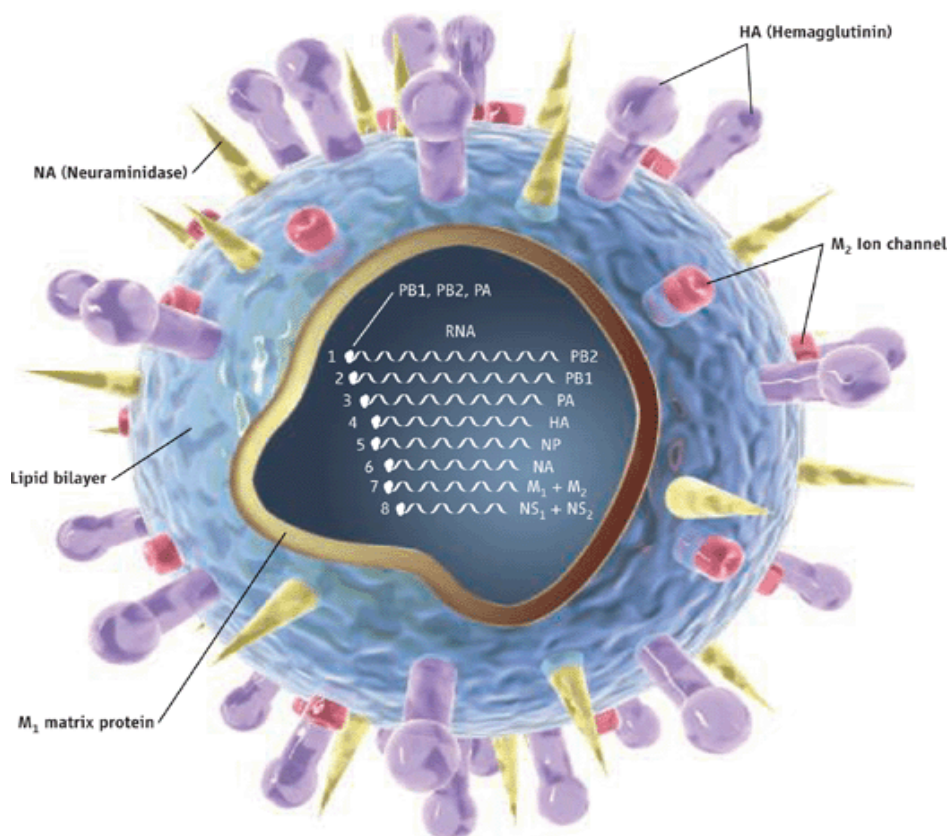
“Influenza has long been with us; indeed, the name itself refers to the ancient belief that it was caused by a malign and supernatural influence. In Florence during the time of the renaissance, astrologers linked a curious juxtaposition of stars with an outbreak of infection in the city and attributed it to the ‘influence’ of the stars, hence influenza.”(6)

Influenza is mainly spread through droplets as aerosols from the airways, but can also infect through direct contact. Viruses of influenza A and B usually cause epidemics during the winter. The influenza B epidemics are often limited while influenza A epidemics has a tendency to be pandemic. Pandemics with influenza A are seen when new subtypes of the

virus occur in which the population has no immunity to. There has been reported 258 human cases (cumulative number) of avian influenza A/(H5N1) to the WHO (World Health Organization). Of these cases there are 153 deaths (7), giving a death rate of 59 %.

A calculation from WHO indicates that influenza gives considerable disease in 3-5 million people annually. It is presumed that the influenza causes casualties in 250.000 – 500.000 people in the industrialized world alone. Even though the infection of influenza limits itself, there are few other diseases that inflict the society so big costs in form of absence from work due to illness, suffering, visit at the physicians office, hospitalization, and direct economic losses (8).

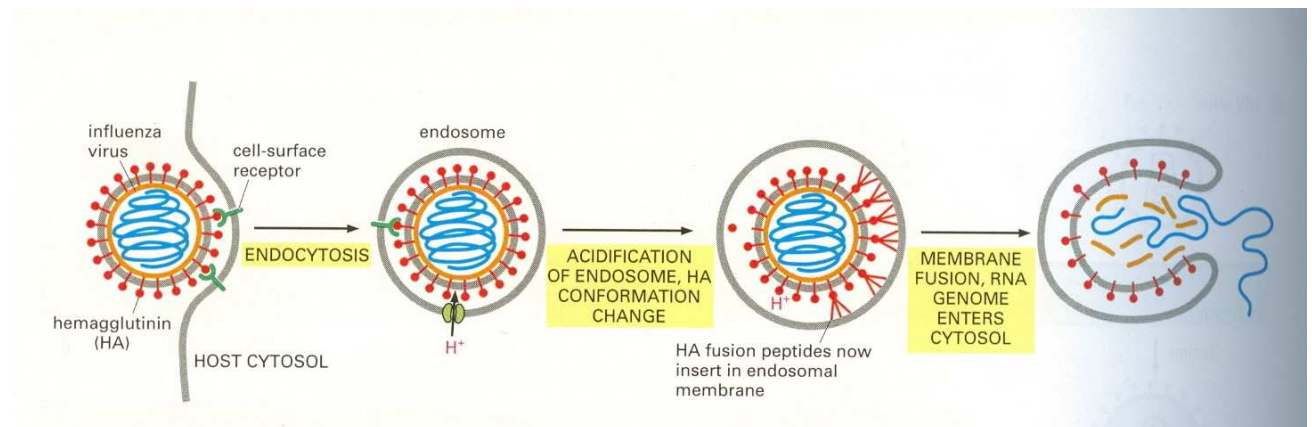
### 5.3.1 Hemagglutinin and neuraminidase



**Figure 3: A model of the influenza virus. Hemagglutinin is showed as purple "mushrooms" and neuraminidase as yellow spikes.**

Hemagglutinin (HA) and neuraminidase (NA) are two proteins located on the surface of the influenza virus as showed in Figure 3. To date, there have been identified 16 subtypes of HA and nine subtypes of NA from influenza A viruses in birds (9). The genome of the influenza

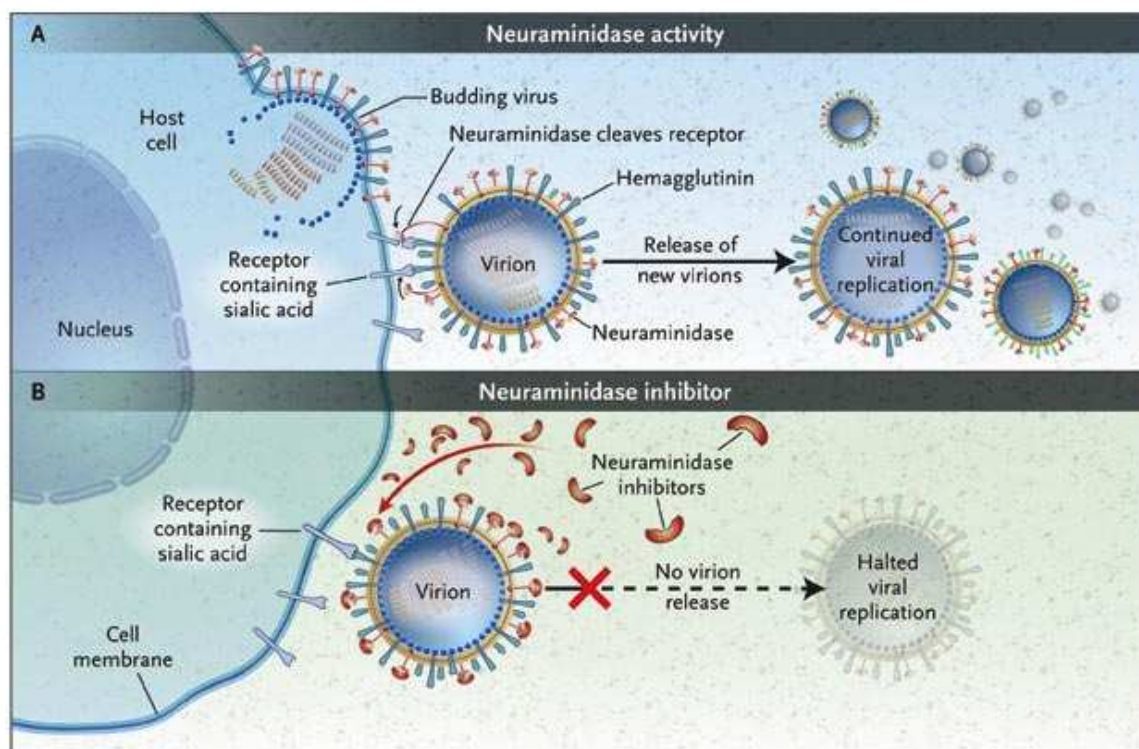
A virus consists of eight RNA molecules, and HA and NA are coded on separate RNA strands. This segmentation of genome facilitates the virus to transfer one subtype of HA and NA from one virus to another during antigenic shift (explained in section 5.3.2 on page 16). These two proteins are antigens recognized by the human body, and the different combinations of the two antigens define the particular strain of influenza. The strains are hence named after which subtype of antigen expressed on its surface. H is the abbreviation for HA and N is the abbreviation for NA e.g. H5N1 (the avian flu threatening us today), H3N2 (the Hong Kong flu in 1968), H2N2 (the asian flu in 1957) and H1N1 (the Spanish flu in 1918). It is only in influenza A that the numbering of HA and NA is used since there has not been observed such variation in the influenza B virus.



**Figure 4: HA complexes with receptors on the cell surface and the virus-receptor complexes are endocytosed. H<sup>+</sup> enters the endosome through an M2 ion channel. The low pH alters the HA in such a way that a fusion peptide is moved to the endosomal membrane. The fusion of the viral and endocytotic membrane result in the release of viral genome into the cell. Reprinted from (2).**

HA plays an important role in the attachment to the terminal sialic acid residues on the cell surface of the new host cell glycoprotein and glycolipids. In addition, HA is involved in the viral fusion with the cell membrane, which results in the intracellular release of the viral contents as outlined in Figure 4 above.

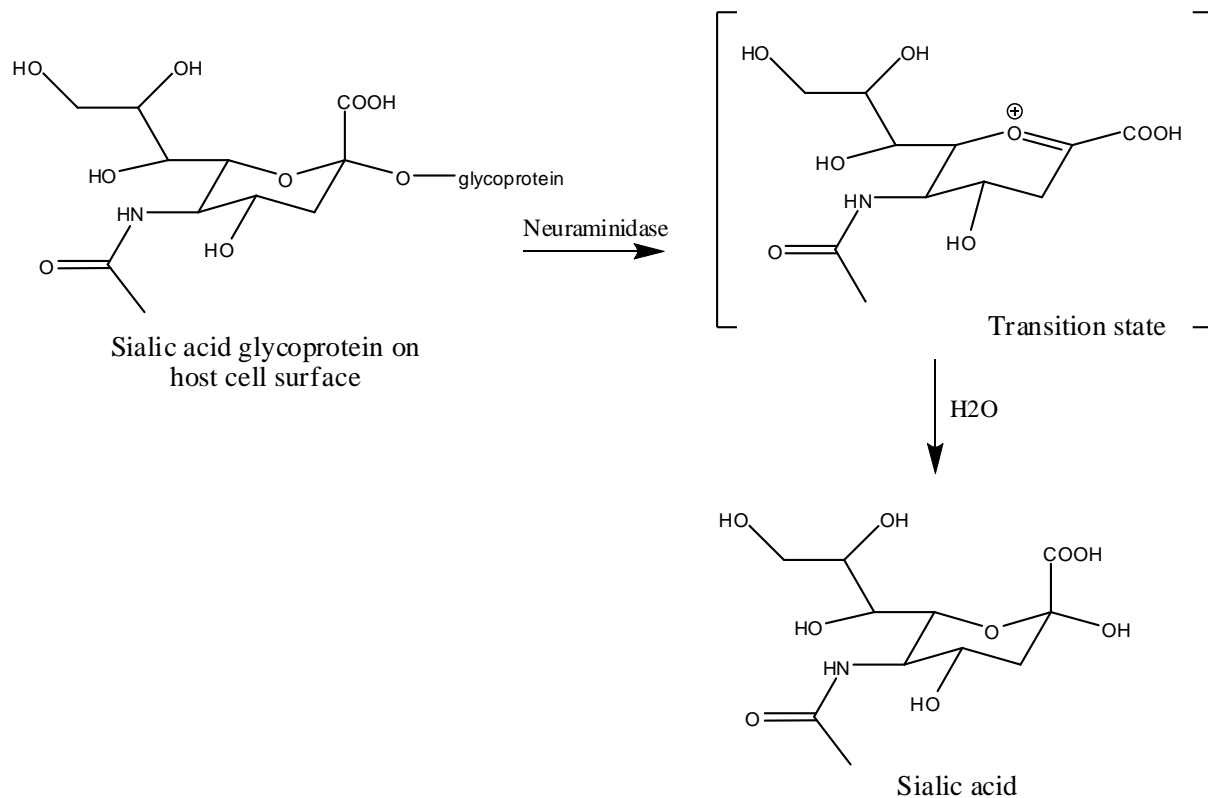




**Figure 5:** Panel A shows action of NA in the continued replication of virions in influenza infection. The replication is blocked by NA inhibitors (Panel B), which prevent virions from being released from the surface of infected cells. Reprinted from (10) with permission from the publishing division of the Massachusetts Medical Society.

NA is an enzyme that cleaves the terminal sialic acid residues of the cellular receptor, and hence is crucial for the release and spread of new progeny virions as displayed in Figure 5 (11). In addition to be important in the release process, a study has proposed that NA in addition to HA is an important factor for the viral entry into the host cell (12).

In the process where NA cleaves the bond between the glycoprotein and sialic acid, there is an intermediate step where the sialic acid is in a transition state as shown in Figure 6 on page 16. This intermediate transition state, which is a half-chair conformation (13), has been the basis for the development of inhibitors for NA. Transition state analogues are regarded as good templates for synthesizing new inhibitory drugs. If a mutation occurs in such a way that it causes drug resistance against NA inhibitors, the intermediate transition state would not fit either, leaving the protein nonfunctional and not virulent. This is actually the case for OTVC which closely resembles the transition state of the sialic acid cleavage. Where mutations in the NA have occurred in a way that the  $IC_{50}$  of OTV is significantly increased, the virus is not virulent and the patients recover from the infection.



**Figure 6: The cleavage of sialic acid from glycoprotein. The transition state is a half-chair conformation.**

### 5.3.2 Antigenic shift and drift

When two or more strains of influenza A infect the same cell, a reassortment of genome (exchange of genes) may occur (14). Such reassortments may yield major genetic changes, and are referred to as antigenic shifts. The ability of antigenic shift has been seen in the influenza A only, not in the influenza B or C (5). Minor genetic changes, on the other hand, are mainly due to point mutations, and are referred to as antigenic drift. The nucleic acid replication by the virus-encoded RNA-dependent RNA polymerase complex is relatively error prone, with a point mutation at about every thousand base per replication cycle (15). Due to Darwins theory of evolution, the natural selection favors the human influenza strains that are able to avoid being neutralized by antibodies from prior infection or vaccination. An infection of a new viral subtype (antigenic shift) can cause a pandemic outbreak such as the Asian flu in 1957 and the Hong Kong flu in 1968, while a reinfection of the same viral subtype with minor genetic changes (antigenic drift) accounts for the annual nature of flu epidemics (11). This also explains the reduced efficacy of influenza vaccination, since the amino acid sequence used in the vaccine may have changed due to the antigenic drift before the epidemic outbreak.



### **5.3.3 Treatment of influenza**

The prophylactic treatment is to vaccinate vulnerable patient groups (e.g. elderly, persons with chronic airway or cardiac diseases, immunocompromised, diabetics and residents in retirement- and nursing homes). This yields a protection of 60–90% in those who are vaccinated (8). However, the supplies of vaccines for mass immunization are in general limited due to inadequate production capabilities (14). It might also be a concern that if the production of vaccines is delayed, the delivery to the markets may be too late to immunize the intended patient groups before the onset of the epidemic.

For those infected with the virus, an early treatment (within two days) with NA inhibitors will shorten the time of illness with 1–4 days, and reduce the frequency of complications (8).

#### **5.3.3.1 Use of antiviral agents**

OTV is indicated for treatment and prophylaxis against flu in persons older than one year. Zanamivir has the indication for treatment in adults and children older than 12 years. The indication for prophylaxis is still not approved in Norway, but is so by the FDA in the USA.

A disease protecting effect has been reported for both OTV and zanamivir to be 70–90 % used prophylactic before or just after infection. When treatment is started within 48 hours after the first symptoms (fever and chills) have appeared, the duration of the disease are shortened with 1–2 days in average. Treatment started within the first 12 hours after onset of fever, shortened the duration of the disease by 3 days. No studies have so far been large enough to prove any mortality rate.

The use of NA inhibitors are the only available treatment against H5N1 avian flu if it should become a pandemic influenza, since H5N1 strains has proven to be resistant to adamantanes (16).

### **5.3.4 From avian flu to human influenza**

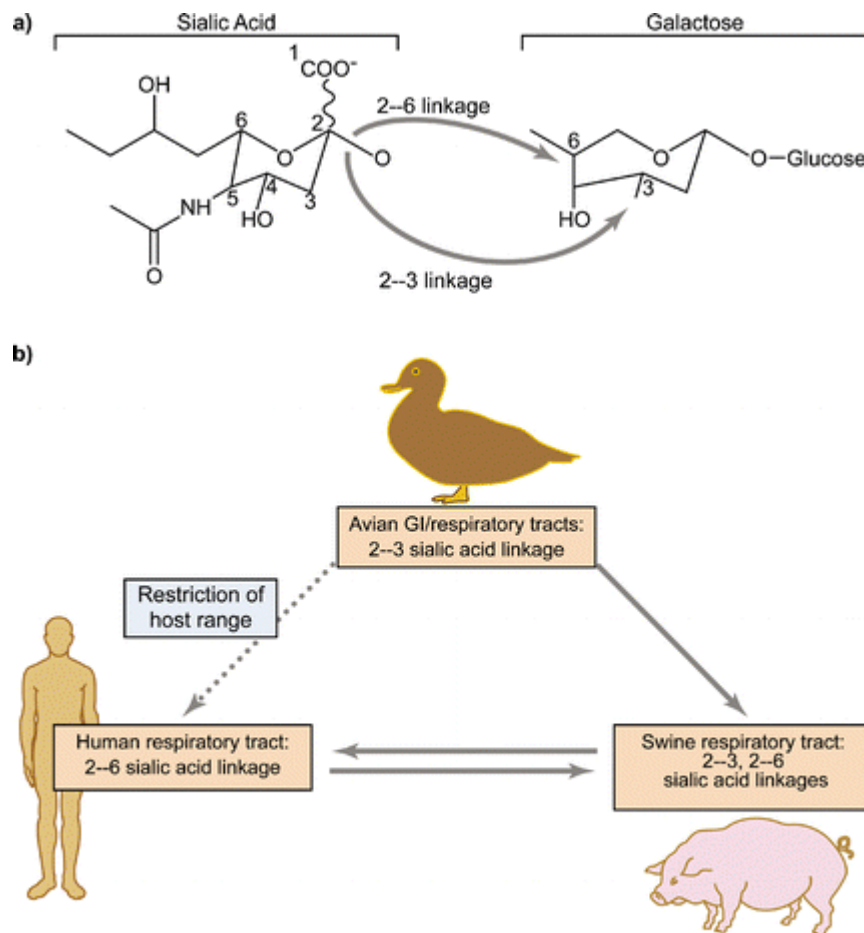
Influenza A viruses, with the most of the HA and NA subtypes, are carried asymptomatically in the gastrointestinal tract of wild birds. Although in wild birds they cause no symptoms,

they do cause disease in domestic birds and mammals, such as pigs and humans. Thus, it can be considered an avian zoonosis (11).

Since the beginning of the twentieth century, only the H1, H2, and H3 subtypes of HA and N1 and N2 subtype of NA have been associated with so stable infection in humans that they result in recurrent annual epidemics. However, this does not mean that the H5N1 as a pandemic among humans is out of the question.

#### **5.3.4.1 External mixing bowl needed or not?**

For a successful viral influenza infection, it is crucial for the virus HA to bind to the sialic acid on glycoproteins or glycolipids on the host cell surface. This receptor may consist of a terminal sialic acid with a 2–3 linkage [NeurAc( $\alpha$ 2–3Gal] or a 2–6 linkage [NeurAc( $\alpha$ 2–6Gal] to a penultimate galactose residue of glycoproteins or glycolipids (11). The two linkages are shown in Figure 5a on page 15. The tracheal epithelia of birds mainly express the 2–3 linkage of sialic acid, while the corresponding cells in humans express the 2–6 linkage. What is interesting is that the pig tracheal epithelia express both the 2–3 and the 2–6 linkage. This has lead to the mixing bowl theory, where the pig has to be infected by both an avian flu and a human influenza, where both viruses infect the same cell. When this takes place, it is possible that an antigenic shift occurs, with a possible outcome of an avian flu with a binding preference to a 2–6 linkage (the human linkage) instead of a 2–3 linkage (the avian linkage). This is shown in Figure 5b on page 15. These progeny viruses can infect humans and spread among the population resulting in a pandemic flu. Early isolates of the 1957 and 1968 pandemics show that they have a binding preference for the 2-6 linkage, while the H5N1 still prefers the 2–3 linkage. This means that the feared H5N1 virus, as it exists today, mainly is a hazard to the birds. Although people have been killed, the virus is not a pandemic yet.



Lewis DB. 2006.  
Annu. Rev. Med. 57:139-54

**Figure 7: (a)** Sialic acid residues can be covalently attached to galactose residues of integral glycoproteins and glycolipids via either 2–3 or 2–6  $\alpha$  linkages. **(b)** Since the avian epithelial cells express only the 2-3 linkage and the corresponding cells of humans express the 2-6 linkage, there is a barrier between the species. However, the epithelial cells of swine express both linkages and can be coinfectd with both human and avian flu. The avian virus can thus overcome the species barrier, by making a detour by pigs. Reprinted from (11)

There is, however, posted a new theory recently that states that viruses that prefer cells with a 2–3 linkage may infect humans directly, without the previously thought obligatory intermediate stage in pigs. The results showed that human lineage viruses preferentially infected human nonciliated respiratory epithelial cells in culture, which preferentially expressed receptors with 2–6 sialic acid linkages. This was no surprise. What is interesting, is that the viruses from the avian isolates (H5N1), in contrast, preferentially bound to and infected the human ciliated respiratory epithelial cells. These cells are a minority among human respiratory epithelial cells, but still a significant number, and the surprising finding is that they express receptors with 2–3 linkages. These results pose that there is a possibility for viral reassortment (antigenic shift) at sites of respiratory epithelium where ciliated and nonciliated epithelial cells are adjacent, and thus using the human as the mixing bowl instead of the pig (17).

### 5.3.5 Avian influenza drug resistance

The H5N1 avian influenza has shown to be able to rapidly develop resistance to the adamantanes (10). There are naturally occurring strains of influenza in the community resistant to the adamantanes, but there is no evidence for such a resistance to either of the two NA inhibitors before their introduction to the markets (18).

The NA inhibitors have proven that they to a far lesser extent provide the development of drug resistant strains of influenza virus than the adamantanes do. About 0.4 % of the OTV treated adults were host for resistant influenza virus which is somewhat higher than for zanamivir where no resistant virus was found (10). Children seem to host viruses that develop resistance to OTV at a higher rate (5.5%) than the viruses in adult hosts (19). There has also been reported a study where 16% of children treated with OTV carried viruses developing resistance, while 2% carry viruses with a mutation not resulting in resistance (20). It is a serious concern that children tend to host resistant strains, especially since they are important in the spread of influenza in the community (21).

However, there has not been documented any transmission of OTV resistant virus from human to human. In fact, it has been demonstrated that viruses with the most common NA mutation (Arg292Lys, which is arginine → lysine mutation at amino acid 292) leave the enzyme defective, which in turn reduces the infectivity and transmissibility of the virus (22). This is most likely because the drug is a transition state analogue. When the drug does not fit, neither do the transition state of the sialic acid.

For the N2 subtype of NA the observed mutations were Arg292Lys, Glu119Val, and Asn294Ser, which were about  $10^4$ – $10^5$ -fold, 500-fold and 300-fold more resistant, respectively, than their pre-treatment NA (20).

## 5.4 Protein binding

### 5.4.1 Drugs and plasma proteins

After the drugs have been absorbed from the gastro intestinal tract, they are transported by the bloodstream to other compartments of the body. If the drug is lipophilic, it has to bind to proteins to be transported by the bloodstream, but if the drug is easily soluble in water it needs no protein and can travel as a free drug.

It is the free drug concentration that is the important parameter for both effect on the target organ and excretion from the body. The drugs bound to proteins are not available for exerting its function to the intended target, nor are they available for excretion. The kidneys only filtrate what is dissolved in blood, so what is bound to proteins easily pass the renal glomerular filtration entering the systemic circulation again. Not only do they avoid renal excretion but also the hepatic conjugations, since the protein bound drugs are not exposed to liver enzymes. There are however exceptions; both the liver and the kidneys have active transporters that effectively can remove compounds bound to proteins (23, 24). However, it is assumed that this is not the case for the prodrugs synthesized in this work.

As the protein bound drugs avoid both the renal and hepatic excretion, it stays longer in the bloodstream, resulting in an increased duration of effect, due to the depot effect of albumin bound prodrug. Thus, increased protein binding will result in prolonged presence of the drug in blood. You can say that the protein functions as a depot for the drug (25).

The distribution volume ( $V_d$ ) is defined as “the volume of fluid required to contain the total amount,  $Q$ , of drug in the body at the same concentration as that present in plasma,  $C_p$ ” (3). Drugs that bind outside the plasma compartment, or enter the body fat, increase the  $V_d$  beyond the total body fluid, which is about 0.55 l/Kg. A high  $V_d$  tells us that the drug penetrates to the tissue compartment, while a drug with low  $V_d$  is confined to plasma compartment and may be highly protein bound.

$$V_d = \frac{Q}{C_p}$$

**Formula 1: The distribution volume ( $V_d$ ) is the ratio of total amount of drug and concentration of free drug in plasma.**

The disadvantage of a highly protein bounded drug is the risk of interaction with other drugs. If other drugs or ligands bind to the same site, the drug with the lowest affinity will be displaced from the protein. This will cause an extraordinary increase of free drug, which may lead to intoxication. However, this happens rarely and only with drugs with a small therapeutic index and with drugs with a relatively high dosage.

### 5.4.2 Albumin

The most abundant protein in plasma is albumin, which accounts for about 60% of all proteins in this body compartment. The albumin binds a broad spectrum of compounds, and those who are strongest bound are hydrophobic organic anions of medium size, long chain fatty acids (LCFA) of 100 – 600 Da, hematin and bilirubin. Examples of compounds that are less strongly bound are tryptophan and ascorbic acid (26).

The “assignment” of albumin in plasma is to solubilise and transport compounds that otherwise are insoluble in water. Since the protein is in abundance compared to its ligands, it can also function as a depot as mentioned above, making the ligands available in plasma way beyond their solubility. Another function of albumin is to cleanse the plasma from toxins, capturing them and transport them to the liver or kidney for excretion.

Albumin tends to bind hydrophobic compounds carrying a negative charge well (25, 27). Among the broad spectrum of cargo the albumin can carry, the most important ones are the endogenous substances, which bind to an adaptable hydrophobic pocket with a positive charge situated at a lysyl or arginyl residue. The negative charge at the ligand forms a strong salt bond with the positive charge from the albumin (26).

In 1975, Sudlow described to binding sites at albumin that were named Sudlow’s site I and II. In this text they are referred to as site I and site II. Many other sites have been described since. Examples of ligands that bind to site I are salicylate, sulphonamides and bilirubin, while tryptophan, thyroxine and octanoate binds to site II. In addition, drugs that are aromatic in nature can also bind to site II. This site can also catalytically hydrolyse various esters. Most of the ligands that bind have one or a few primary binding sites, to which it binds well, in addition to other binding sites with lower affinity. It has been demonstrated that lipids lacking the carboxyl group bind less tightly to albumin than the LCFA.

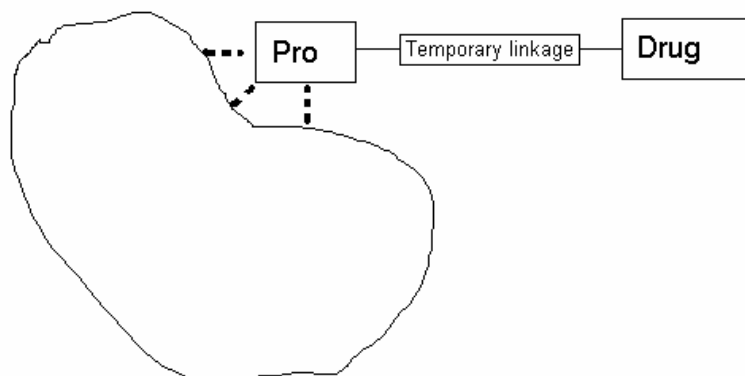
LCFAs, which are fatty acids with carbon chains from C<sub>16</sub> to C<sub>20</sub>, and medium chain fatty acids (MCFA) have several binding sites in albumin. LCFAs, however, seem to have their primary binding site at site II, and MCFAs at site I. The fact that LCFAs primarily bind to site II may pose problems regarding therapy with drugs with the same primary binding site, since LCFAs may displace the drug and cause unpredictable plasma concentrations of the drug. It is hard to predict which patients this will concern, since there can be great inter- and intra individually differences (26).

*MacKichan* has reviewed the problems with drug displacement interactions due to protein binding. There are two ways of displacing a drug from a protein, competitive and non-competitive. In the former case, there is a competition between the displacer and the drug for the same binding site, while in the case of a non-competitive inhibition, the displacer alters the tertiary conformation of the protein, making the binding site unrecognisable for the drug. He states four general requirements that must be fulfilled for a drug displacement to be clinically relevant:

- The displaced drug must be highly bound, so that a given decrease in its bound fraction will result in a large percentage increase in its unbound fraction.
- The displacer and the displaced drug must share a common binding site (if competitive) or a common protein (if non-competitive).
- For true competition to occur, the binding sites must be limited in number, hence, the molar concentration of the displacer must approach the concentration of protein binding sites.
- The free concentration of the displacer must be higher than that of the displaced drug and/or the binding site affinity for the displacer must be higher than that for the displaced drug.

He concluded that “although drug displacement is common and sometimes predictable, it alone is rarely responsible for observations of adverse drug interactions” (28).

### 5.4.3 The reasons for making a protein binding prodrug



**Figure 8: The pro moiety binds to albumin and is temporarily linked to the drug moiety. When the linkage is broken, the drug is a free drug in plasma.**

There are mainly two advantages with protein bounded drugs. First, as mentioned above, the proteins function as a depot for protein binding drugs, due to the depot effect. This means that a frequent dosage is unnecessary. This will significantly ease a higher compliance of drug therapy, which is especially important in HIV patients. Secondly, there has been discovered that cancer tissue has an enhanced permeability and retention effect (EPR), where proteins and lipids are restrained by the tissue. The mechanisms for this effect are “extensive angiogenesis and hence hypervascularity, defective vascular architecture, impaired lymphatic drainage/recovery system, and greatly increased production of a number of permeability mediators” (29). This enhanced vascular permeability has also been seen in infected tissues with plasma proteins, macro molecules and lipid particles leaking into the interstitial space (29). The EPR concept is now regarded the “gold standard” in the design of new anti cancer agents (30). It has been discovered that mice infected with influenza virus carry out excessive production of superoxide and NO, two of the permeability mediators that result in the EPR effect. This indicates that an EPR effect can occur during an influenza infection.

This EPR characteristic is possible to exploit in drug targeting. This has already been done by *Kratz et. al.*, who synthesized a prodrug of doxorubicin, which is the chemotherapeutic agent against several types of cancer, covalently bound to a thiol group at albumin. The prodrug is proposed to be released in close proximity to the cancer cell, due to the acidic environment and the acid sensitive bond between doxorubicin and albumin (31).



If the prodrug is synthesized by extracting albumin in large scale and linking it to the doxorubicin molecule, there will most likely be a problem with an unwanted immune response. Making the drug in situ in a hospital, linking the drug to the patients own albumin might circumvent this problem. However, it is very unpractical and takes a lot of resources to do the extraction, purification and finally the linking of drug to albumin ex vivo.

## **5.5 The prodrug concept**

There are several ways of dealing with poor drug delivery characteristics. Some drug delivery problems can be circumvented by dosage form design. Another approach is to make a new analog of the parent drug. Designing a new drug with the desired physiochemical properties, the pharmacological profile may change, which results in series of new time consuming and expensive biological testing. Delivery characteristics are often changed appropriately by synthesizing a bio reversible chemical derivate. This is called the prodrug approach.

The concept of making bioreversible drugs based on other drugs that already are known to be effective has lately become a popular way of doing drug research. In fact, there was a 2000 % increase in new prodrug patents from 1993 to 2002 in the USA. New cancer drugs accounts for 37 % of these patents. In 2001 and 2002, 14 % of all drugs approved by the FDA were prodrugs (32).

The prodrug in itself has no effect pharmacologically, but after degradation in vivo it is split into an active drug moiety (the parent drug) and an inert pro moiety. This provides a number of advantages. First, the physiochemical and pharmacological properties are transient, allowing the prodrug to have favorable properties, which the parent drug lacks, till the in vivo cleavage. This means that it is possible to temporarily mask the undesirable physiochemical properties of the parent drug. Secondly, synthesizing different chemical derivatives of the same drug allows the prodrug to have a broad spectrum of transient physiochemical properties (33). Thus you can pick and choose the properties you want.

According to Claus S. Larsen and Jesper Østergaard, rational prodrug design must consist of three basic steps (33):

- Identification of the drug delivery problem
- Identification of the physiochemical properties required for maximum efficacy or delivery
- Selection of a transport moiety providing a prodrug derivate exhibiting the proper physiochemical characteristics and which can be cleaved in the desired biological compartment

Dissolution and transport processes are the two processes which largely determine how much of the drug reaching its target receptors. These processes primarily depend on lipophilicity and aqueous solubility. The transport rate of a drug is usually enhanced by making the prodrug a more lipophilic substance. Initially the transport rate over membranes is increased exponentially with increased lipophilicity. However, this is only to a certain point. Drugs or prodrugs with too high lipophilicities often have low bioavailability due to the poor aqueous solubility. On the other hand, drugs that are too hydrophilic, often exhibit poor transport properties. Therefore, it is apparent that the drug or prodrug must possess both of the physiochemical properties to a certain degree (33).

Reasons for making a prodrug (33):

- Drug targeting
- Stabilization of the drug
- Enhancement of drug solubility
- Improvement of circulation life time and hence extended duration of action

Drug targeting is the main reason for making prodrug of OTV. This is due to the enhanced protein binding and the EPR effect in inflamed tissue, which is discussed in section 5.4.3 on page 24. Increased protein binding also results in extended duration of action due to the depot effect of decreased renal filtration and hepatic conjugation as outlined in section 5.4.1 on page 21.

## 5.6 Other antiviral agents

Since the viruses are intracellular parasites utilizing the hosts machinery for replication, it is difficult to find a chemical that halts or slows the viral infection. Chemical agents that inhibit both the host and the virus are not a good choice of therapy, due to the adverse effect they might cause. The best approach is to identify the unique functions of the virus, which differ significantly from the functions of the host, and then block this critical step in its life cycle (5). Table 2 lists some targets for antiviral drugs and examples of available medicines in each group.

**Table 2: Some targets for antiviral drugs. Reprinted and modified from (5).**

<b>Step in virus life cycle targeted</b>	<b>Molecular target of inhibitor</b>	<b>Example</b>
Virus attachment and entry	Surface protein-receptor interaction	Receptor analogues, fusion protein amantadine
DNA virus genome replication	Viral DNA polymerase	Acyclovir
RNA virus genome replication	Viral RNA replicase	(Theoretical)
Retrovirus – reverse transcription	Reverse transcriptase	AZT, ddC, ddI
Retrovirus – integration	Integrase	(Theoretical)
Viral transcriptional regulation	HIV <i>tat</i>	(Theoretical)
Viral mRNA posttranscriptional processing (splicing)	HIV <i>rev</i>	(Theoretical)
Virion assembly	Viral protease	Protease inhibitors (ritonavir, atazanavir)
Virion assembly	Capsid protein – protein interactions, budding	Rimantadine, protease inhibitors

### 5.6.1 Reverse transcriptase inhibitors and protease inhibitors

These two classes of antiviral agents are the most important in treating an infection of HIV. Reverse transcriptase is an enzyme that makes a double stranded DNA copy of the viral RNA after the entry into the cell. This DNA then enters the cell nucleus where it integrates with the host DNA. When the hosts DNA is transcribed, a provirus mRNA is transcribed at the same time. The host ribosomes translate the provirus mRNA into biochemically inert polypeptides.

The enzyme protease cleaves the polypeptides at the appropriate places to produce structural and functional proteins. Since protease does not occur in the host, it is a good drug target. An inhibition of both of the enzymes reverse transcriptase and protease is a critical loss for the viral replication. However, these inhibitions do not kill the virus, since it remains dormant in the host nucleus, incorporated into its DNA.

The reverse transcriptase inhibitors are divided in two groups; nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI).

The NRTIs are converted by the host cell enzymes to the active compounds by phosphorylation. These compounds compete with the substrates for reverse transcriptase and the incorporation of the compound results in the termination of the growing viral DNA chain. There are currently available 6 drugs in the NRTI class.

The NNRTIs are no substrate for the enzyme, but binds near the catalytic site denaturing the protein. There are currently available two drugs NNRTIs.

The best treatment is a combination of several drugs, known as highly active antiretroviral therapy (HAART), which consists of a cocktail of some of the drugs mentioned above. A typical HAART combination would consist of two NRTI with either a NNRTI or one or two protease inhibitors (3).

### **5.6.2 Acyclovir and valacyclovir**

Acycloguanosine (prescribed under the name acyclovir) was the first nucleoside analogue that was a chain terminating inhibitor. After acycloguanosine is transported into the cell and triphosphorylated, it is utilized as a substrate for the herpes virus DNA polymerase. The drug lacks the 3'OH group, which results in the termination the growing DNA chain. The herpes virus encodes a protein called thymidine kinase (TK), which is required for the first phosphorylation of acycloguanosine. The drug will inhibit the viral DNA polymerase about 10 times more efficiently than the corresponding cellular enzyme. However, since the activation of the drug requires a viral enzyme, it is non-toxic to uninfected cells (5).

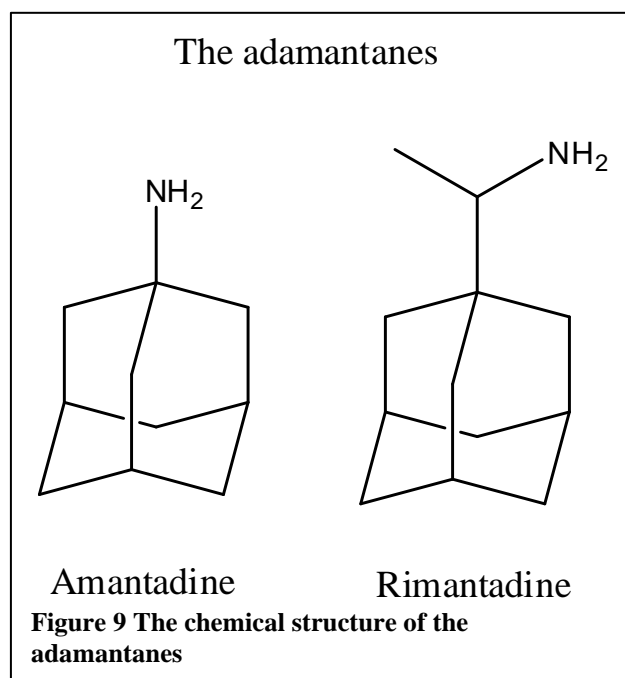
### **5.6.3 Zanamivir**

Like OTV, zanamivir is an inhibitor of NA, and inhibits the spread of progeny virions from the infected cell. Zanamivir binds to NA close to its active site, inhibiting the natural substrate, sialic acid, isosterically from binding to the enzyme (13). If an outbreak of H5N1

pandemic influenza should occur, the only possibility for treatment and prophylaxis is the use of either zanamivir or OTV (16). Due to poor bioavailability orally, the drug is administered pulmonary as a dry powder. Since it is easier to swallow a mixture or a capsule than fumble with an inhalator, Tamiflu (OTV) has reached higher sales than Relenza (zanamivir). An inhalation of zanamivir could pose a problem for a patient suffering from some of the respiratory symptoms of influenza (34).

#### 5.6.4 Adamantanes

This group consists of two drugs; amantadine and rimantadine (Figure 9), none of them are on the market in Norway. Both are used against influenza A. After the HA has bound to the sialic acid and the virus has undergone an endocytosis, the pH lowers to around 5. At this pH the membrane of the enveloped virus undergoes a fusion with the viral membrane, resulting in the entry of the viral nucleocapsid (5). The adamantanes work by interfering with viral uncoating inside the cell. The drug is a basic primary amine that inhibits the M2 ion channel that is responsible for the acidification of the resulting endosome after viral entry (35). In this way, the adamantanes will counteract the acidification that is crucial for the complete viral entry.



“[The adamantanes] are effective only against influenza A and are associated with several toxic effects and with rapid emergence of drug-resistant variants.”(10) Isolates from the adamantane-resistant strains have shown to be genetically stable. They are as pathogenic as the wild type influenza, and can be transmitted between people. These characteristics have limited the use of adamantanes (10). This is probably why the adamantanes are not registered in Norway. In fact, the avian H5N1 from 2004 has shown to be resistant to adamantanes (16)

## 5.7 Oseltamivir and its new successor

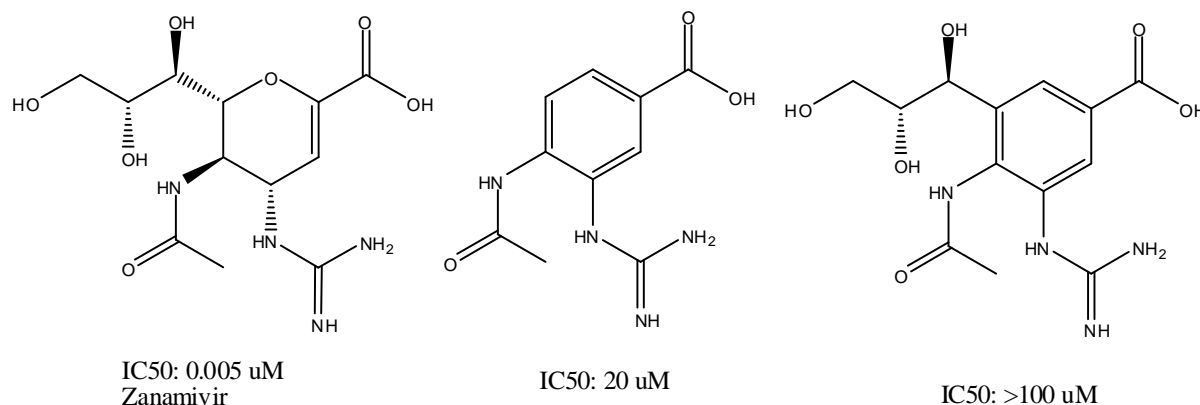
OTV is marketed in Norway as Tamiflu®, and has been approved by the Norwegian Medicines Agency to treat influenza in infected persons over one year of age. The treatment has to be started within 48 hours after the first symptoms appear, but is more effective the earlier the treatment begins.

It has been shown higher virulence among recent isolated H5N1 viruses reducing the efficacy of NA inhibitors. OTV in a higher daily dose and a longer duration of treatment has improved the survival of mice (36). This finding suggests that a higher dose of OTV may be necessary for human avian influenza than what currently is recommended. However, more research in this field is needed.

### 5.7.1 The development of oseltamivir

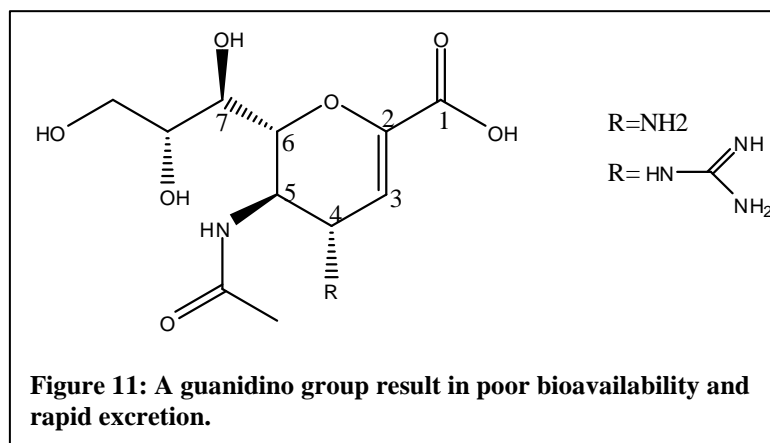
The development of this drug is a good example of how to make a new chemical entity based on rational information available from the crystal structure of NA and the complex with its inhibitors.

The structure of zanamivir is a half-chair conformation, and thus is almost flat. This gives the substituents of the ring an almost equatorial orientation (Figure 6 on page 15). There have been attempts to synthesize aromatic analogues of sialic acid, which were thought to have inhibitory effect on NA. Although the aromatic compounds showed lower inhibitory activity than zanamivir (Figure 10 on page 30), this work has given us the knowledge that the substituents of the ring cannot be totally equatorial. This proves that a good inhibitor must comply with the strict demands of the shallow binding pocket of the influenza NA (37).

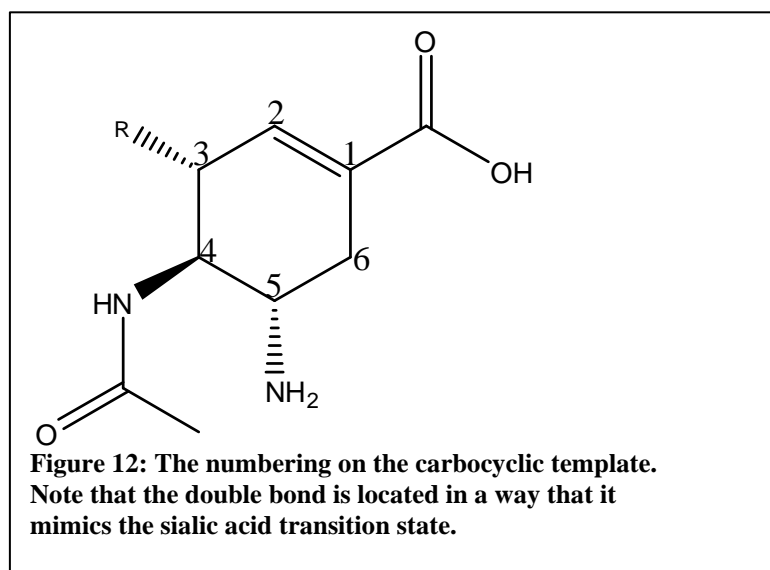


**Figure 10: The inhibitory effect of two aromatic compounds on the NA enzyme.**

A dissertation has recently confirmed these results. Other aromatic inhibitors were synthesized and evaluated for a Ph. D. degree, and they all showed  $IC_{50}$  values higher than OTV (38). Both the amino group (as in OTV) and the guanidino group (as in zanamivir) in 4-position on the structure on the right (Figure 11) forms salt bridges with Glu119, but only the guanidine group provides the molecule poor bioavailability and rapid excretion. These properties rule out the drug from being administered orally, as is the case for zanamivir.



An idea of locating the double bond in the structure on the same spot as in the transition state has been of great value in the development of the new NA inhibitor. As a template, it was used a carbocyclic ring instead of the dihydropyran ring of the Neu5Ac2en system. The carbocyclic ring is chemically more stable than the dihydropyran ring and is easier to modify for antiviral optimization. This ring has another numbering than Neu5Ac2en due to another localisation of the double bond (Figure 12 to the right), which structurally has a closer resemblance with the sialic acid transitionstate during the hydrolysis in the



nuraminidase. (Figure 6 on page 16). This resemblance has proven to be essential regarding NA inhibitory activity (39). When a lipophilic group was attached to the carbon in C3 position in the carbocyclic ring, the NA inhibitory activity increased, indicating that there is a lipophilic pocket in the glycerol binding site of the enzyme. This was confirmed by x-ray crystallography (39).

It was discovered that the C<sub>7</sub> hydroxyl does not interact with any amino acids in the active site of NA (40), which lead to the conclusion that this group could be eliminated from the structure. As a matter of fact, there is a hydrophobic pocket.

The double bond in the sialic acid transition state is polarized and electron deficient.

Replacing the C<sub>7</sub> carbon with an oxygen atom, will further contribute to the mimic of the

transition state since the oxygen will “reduce the electron density of the double bond via the  $\sigma$  bond electronegative effect” (39). A crystallographic study where OTVC were bound to the NA (as shown in Figure 13) showed that the carboxylate group is surrounded by three arginyl residues and the C<sub>4</sub> hydroxyl group (which is substituted amino group in OTV and guanidine group in zanamivir) were placed in a pocket with the highly conserved residues Glu 119 and Glu 227. There was also a hydrogen bonding between the protein and both the amide nitrogen and carbonyl oxygen at the N-acetyl group located in the C<sub>5</sub> position, while the methyl group was seated on a hydrophobic patch (39).

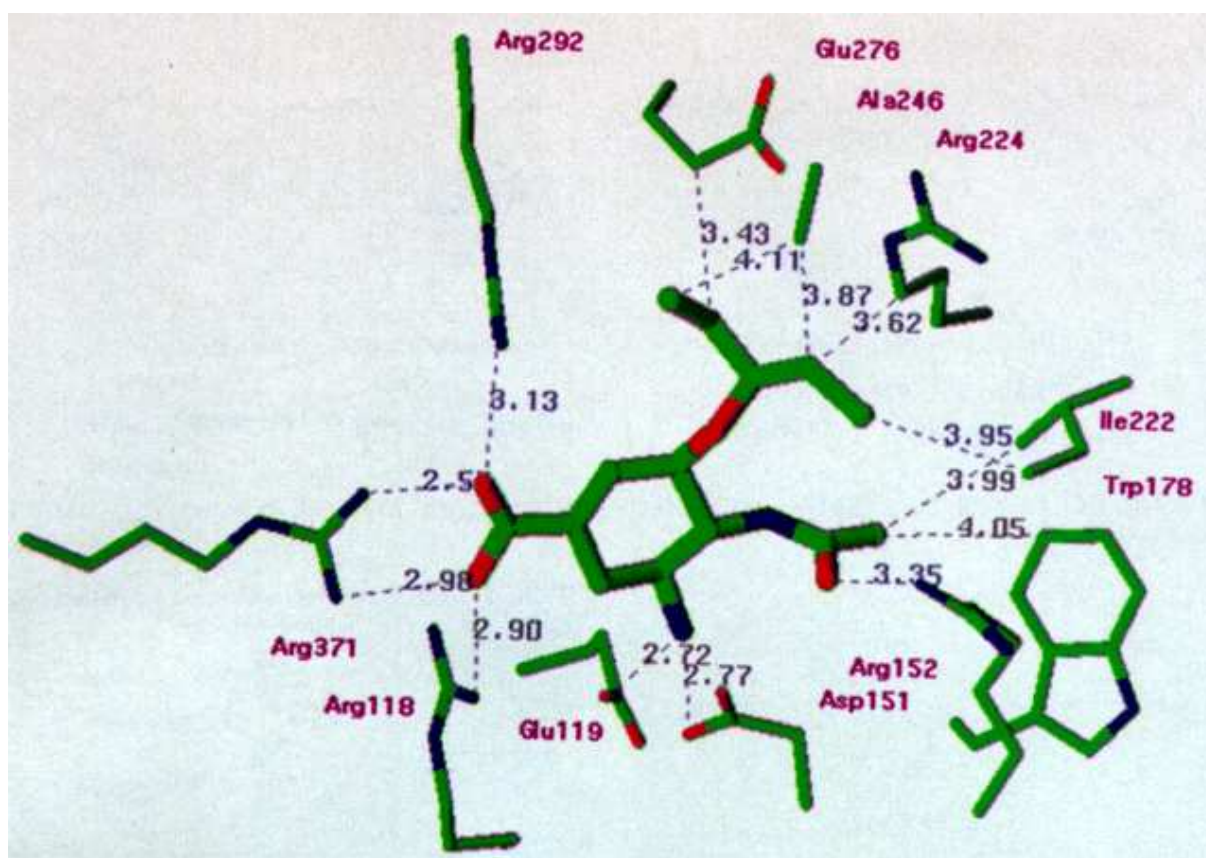


Figure 13: The interactions of OTV in the NA enzyme. Reprinted from (26)

There has been performed an experiment on introducing lipophilic substituents (Cl, SCH<sub>3</sub>, and CH<sub>3</sub>) in the C<sub>2</sub> position (Figure 14) and the inhibitory characteristics were investigated. All three compounds proved to be poor inhibitors of NA due to the IC<sub>50</sub> values of 3100, 3400, 2300 nM respectively. This result indicates that the hydrophobic pocket at this place in the enzyme has a

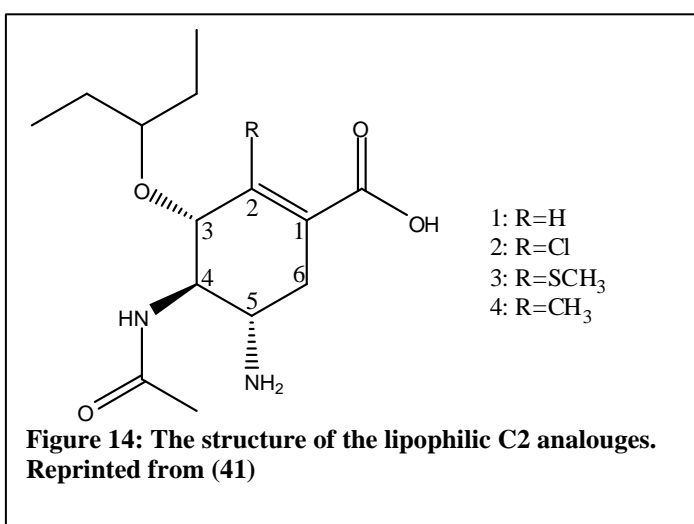


Figure 14: The structure of the lipophilic C2 analogues. Reprinted from (41)

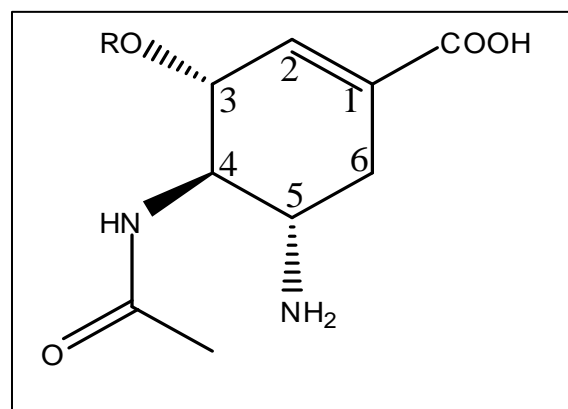


limited space that does not allow for other atoms than hydrogen to bind (41).

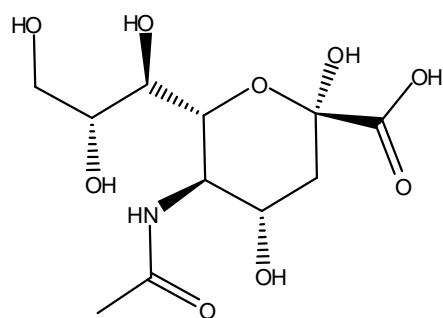
The backbone of the glycerol of sialic acid interacts with the hydrophobic Arg 224. This was the reason for the search for the optimum lipophilic analogues for the C<sub>3</sub> side chain. Several structures were synthesized with such substituents in the C<sub>3</sub> position, and analyzed with respect to the IC<sub>50</sub> values shown in Table 3 below (39).

**Table 3: The IC<sub>50</sub> values for the various lipophilic substituents is C3 position. Reprinted with modifications from (39).**

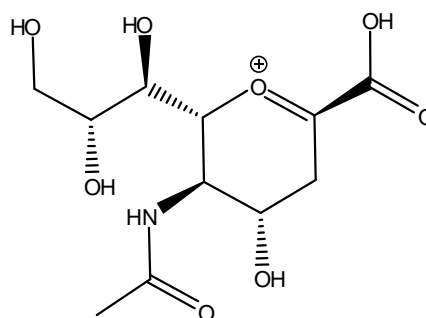
R	IC <sub>50</sub> (nM)
H	6300
CH <sub>3</sub>	3700
CH <sub>3</sub> CH <sub>2</sub>	2000
CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub>	180
CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub>	300
(CH <sub>3</sub> ) <sub>2</sub> CHCH <sub>2</sub>	200
CH <sub>3</sub> CH <sub>2</sub> (CH <sub>3</sub> )CH* (R-isomer)	10
CH <sub>3</sub> CH <sub>2</sub> (CH <sub>3</sub> )CH* (S-isomer)	9
(CH <sub>3</sub> CH <sub>2</sub> ) <sub>2</sub> CH	1
(CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> CH	16



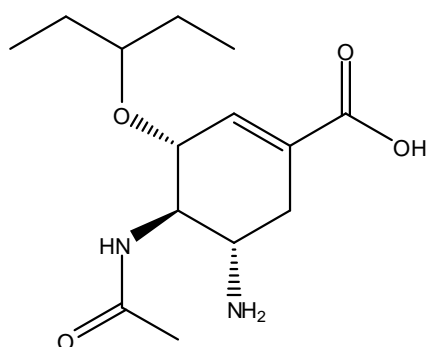
The results as displayed in Table 3 above show an increasing affinity to the enzyme when the aliphatic carbon chain expands up to n-propyl, but further expansion decreases the affinity. Geometry also seem to be of importance, since branching of the  $\alpha$  carbon, as in OTV, yields an IC<sub>50</sub> of 1 nM, while branching of the  $\beta$  carbon gives an IC<sub>50</sub> of 200 nM. The size of the branching is also an important characteristic as the 4-heptyl substituent is a weaker (IC<sub>50</sub>=16) inhibitor than the 3-pentyl substituent is with an IC<sub>50</sub> of 1 (39).



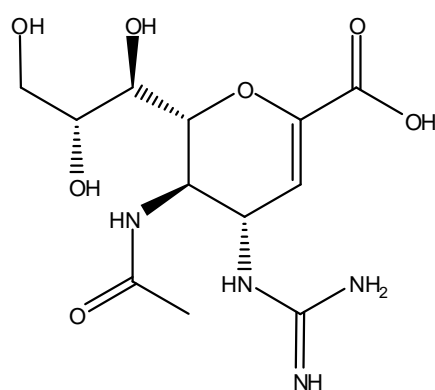
sialic acid



sialic acid transition state



Oseltamivir carboxylate



Zanamivir

**Figure 15: The chemical structure of sialic acid (the substrate for NA), its transition state inside NA and the NA inhibitors OTVC and zanamivir.**

## **6 AIM OF THE THESIS**

### **6.1 *Main aim***

The main aim of this study is to synthesize new novel compounds that are prodrugs of OTVC, with increased protein binding compared to the parent drug. The novel prodrugs should also have a slower conversion rate to OTVC than OTC.

### **6.2 *Sub aims***

#### **6.2.1 Synthesis strategy**

A rational strategy for the synthesis shall be worked out. The synthesis shall end with a novel prodrug that is thought to exhibit higher protein binding than the parent compound.

#### **6.2.2 Selection of pro moieties**

A series of pro moieties with slightly different chemical structures shall be selected. The structure should have a negative charge in form of a carboxylic acid in order to bind to albumin.

#### **6.2.3 Crystallographical studies**

Crystals of OTV and OTVC shall be grown, and x-ray structure shall be solved.

#### **6.2.4 Albumin binding**

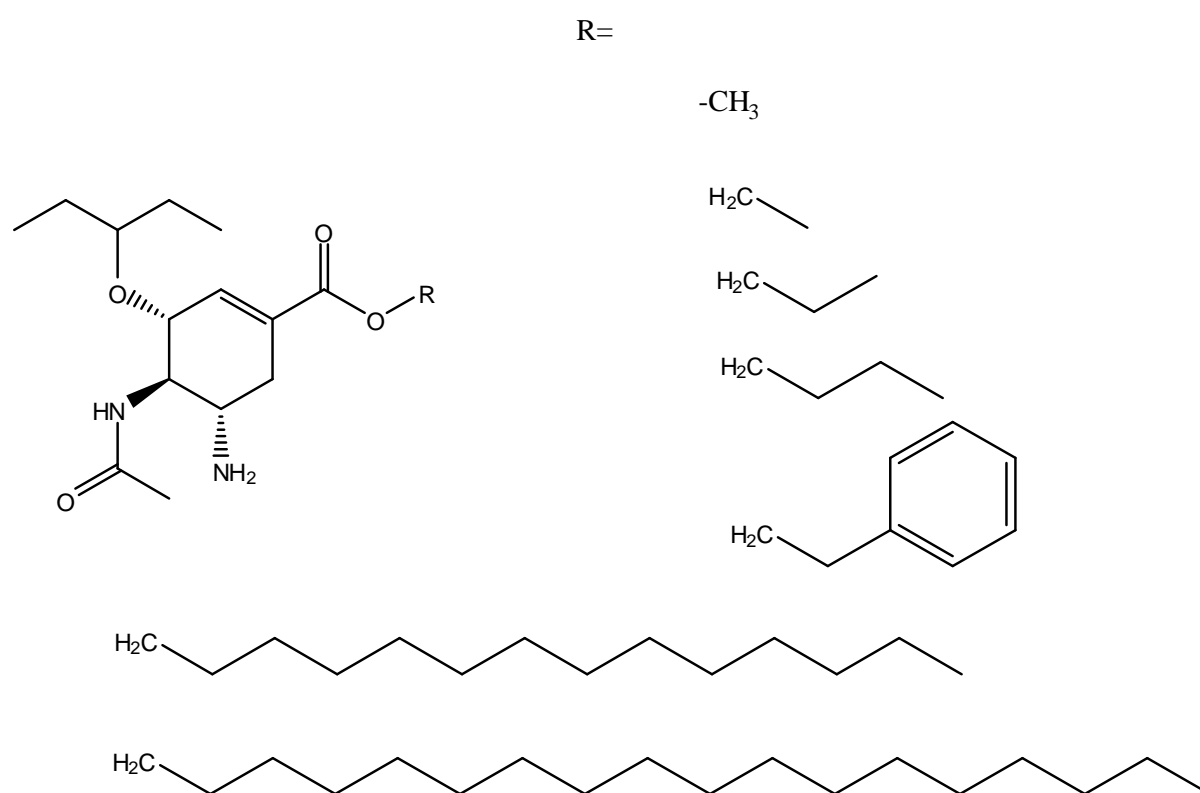
When the syntheses of the prodrugs are done, the binding to human serum albumin shall be carried out. The albumin binding of the prodrug shall be related to the corresponding results from OTV and OTVC. Stability to esterase shall also be determined.

## 7 RESULTS AND DISCUSSION

### 7.1 Synthesis

#### 7.1.1 Background

A search on SciFinder for other synthesized carboxylic acid esters of OTV revealed that there was much work left to do in this area. There has been described a total of 7 esters based on the OTV scaffold. (See Figure 16 below)



**Figure 16: The synthesized carboxylic acid ester structures based on the OTV scaffold.**

The prodrugs synthesized in Figure 16 above will result in increased protein binding due to increased lipophilicity. However, this results in decreased water solubility, and hence the prodrug might be difficult to formulate for oral administration.

By introducing a carboxylic acid group to the pro moiety, the water solubility increases, which facilitates the oral drug formulation. In addition, the protein binding may also increase due to the cationic charges on the albumin as discussed in section 5.4.2 on page 22.

Based on the result from the structure search, we can conclude that this is an unexplored area that needs to be examined.

### 7.1.2 Synthesis strategy

The overall synthesis strategy was to hydrolyze the present ester on OTV, and add a new ligand by esterification. The problem with the esterification in OTVC is that the amino group can react with a carboxylic acid on a neighbor OTVC, producing an amide linked dimer or even a polymer. Thus, the amino should be protected to ensure that the carboxylic acid reacts with the alcohol only.

Generally, purification is easier when the molecule has only an acid or only an amine. If the molecule has both groups, one of them is always charged, thus making the purification difficult. Therefore, it was decided that the amine group should be protected before the hydrolysis to avoid the possible troublesome purification.

The pro moiety of the prodrug, the ligand, contains an acid group that is supposed to interact with the cations of albumin (see section 5.4.2 on page 22), but also an alcohol group that is supposed to be linked by an ester bond to the active substance, OTVC. The problem is that the alcohol can react with the acid of a neighboring ligand, resulting in a ligand dimer, or even polyester of the ligand. To circumvent this problem, the acid of the ligand should be protected in the synthesis.

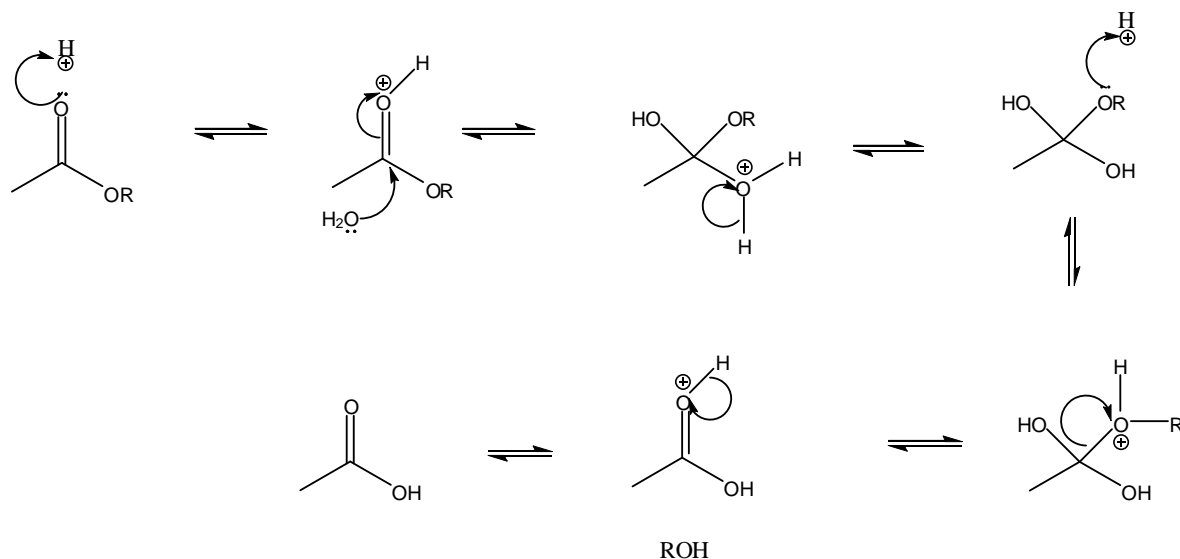
As a protection group for the amine, isonicotinyl was thought to be a good choice. The isonicotinyl group is described to be an especially stabile carbamate protecting group and might not be deprotected during the acidic hydrolysis of the ester. The deprotection of the isonicotinyl group is done with acid in the presence of zinc. However, this synthesis proved to be troublesome. This is discussed in section 7.2.7 on page 51.

Since the conditions for the deprotection of the amine is acid in the presence of zinc dust, it would be advantageous with a protection group for the acid of the ligand that is deprotected under the same conditions. In this way, the deprotection can be performed in one step of synthesis. TCE exhibit this property, and was therefore chosen.

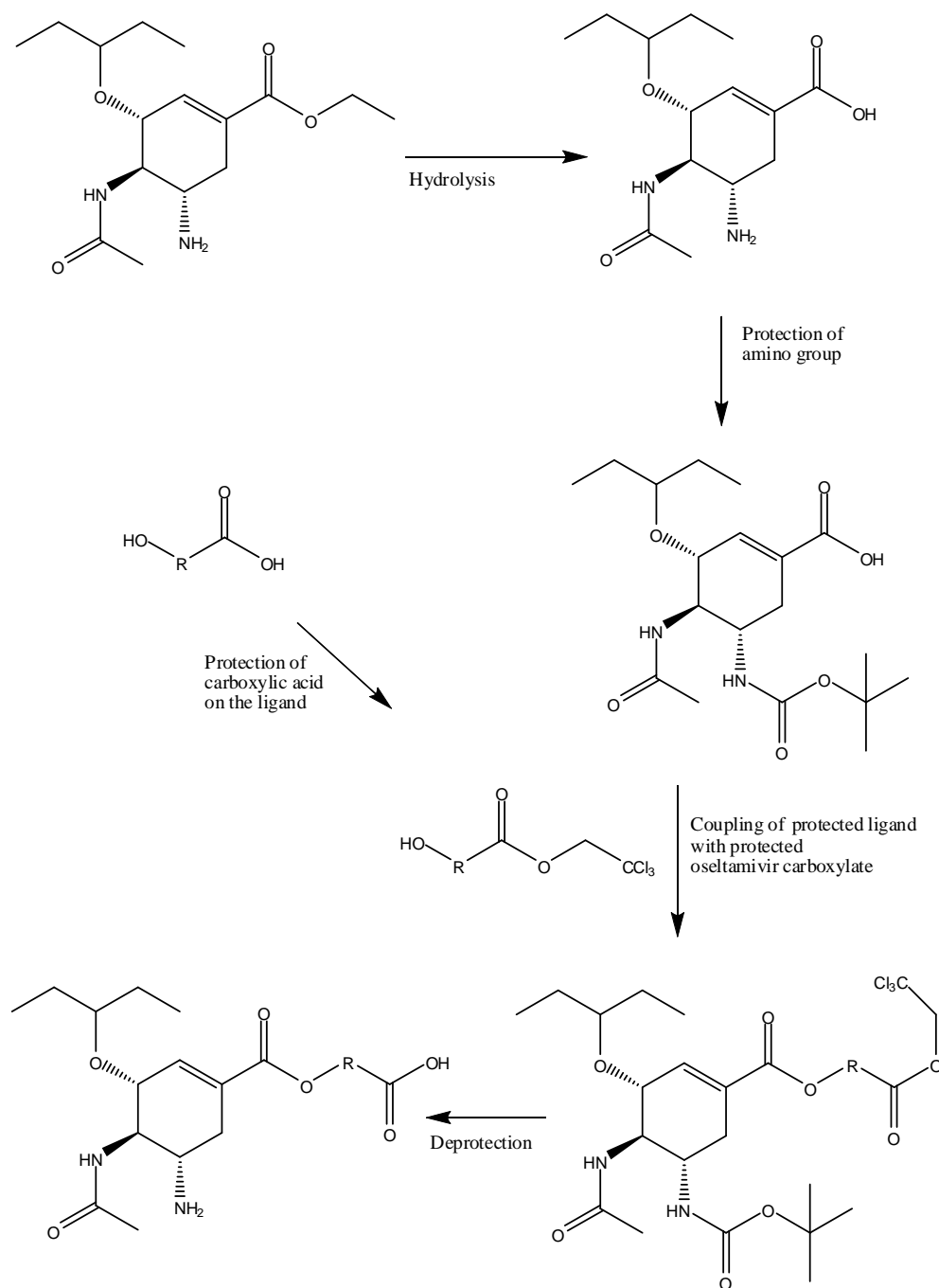
Since the isonicotinyl synthesis proved to be difficult, an alternative synthesis was needed. Protection with Boc cannot be done before the hydrolysis since the group is labile under acidic conditions. However, the protection could be done after the acidic hydrolysis, with the purification in a later step of the synthesis. See Figure 18. The deprotection of Boc are by several described in the literature to be performed under various acidic conditions (42-44).

In order to deprotect both protection groups at the same time, tert butyl alcohol could be the protection group for the acid. Tert butyl alcohol is deprotected under moderately acidic conditions (44). However, at the time where it was discovered that the synthesis of isonicotinyl protected OTV was troublesome, most of the TCE protected ligands were already synthesized. Using acidic conditions in the presence of Zn dust will deprotect the TCE, however Boc will most likely also be deprotected although the presence of Zn is unnecessary.

Although Woodward *et. al.* utilized 90% acetic acid to deprotect TCE in their cephalosporin synthesis (45), it might be feasible to use 100% acetic acid to reduce the risk of nucleophilic attacks of H<sub>2</sub>O on the ester bond between the drug and the pro moiety. With concentrated acetic acid the mechanism in Figure 17 below will not be able to proceed due to the lack of water.



**Figure 17: The reaction mechanism for acidic ester hydrolysis.**



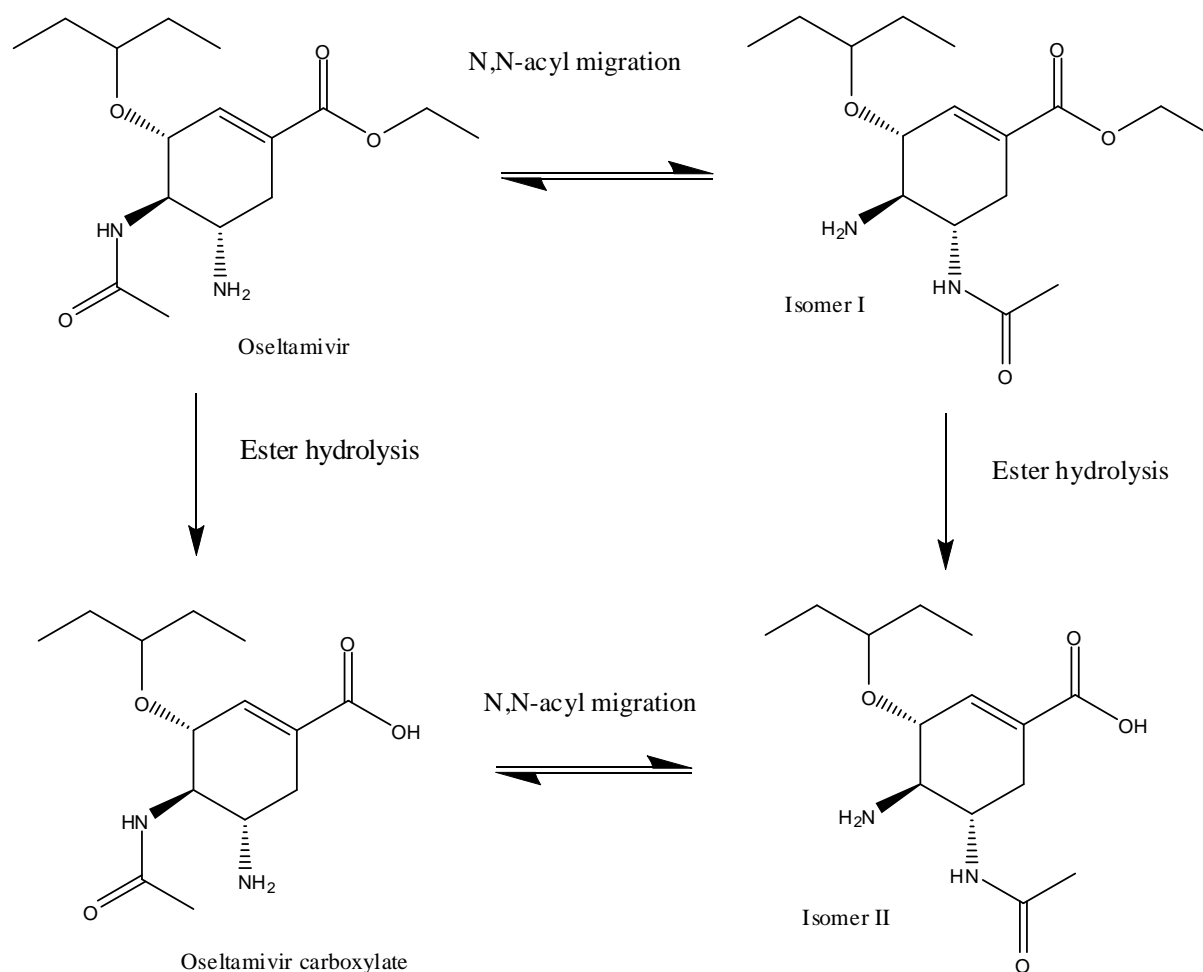
**Figure 18: The final overall synthesis scheme for the synthesis of the new protein binding prodrug. The synthesis involves hydrolysis of OTV, protection of ligand and OTVC, esterification of protected ligand with protected OTVC, and deprotection to produce a new prodrug.**

### 7.1.3 The hydrolysis of oseltamivir

In order to synthesize a new prodrug of OTV, the present ester has to be removed by e.g. hydrolysis. There are described basic hydrolysis of OTV in the literature (41), however, the initial experiments started with testing out acidic hydrolysis, and these results were found

satisfactory. The mechanism for acid catalyzed ester hydrolysis is lined out in Figure 17 on page 38.

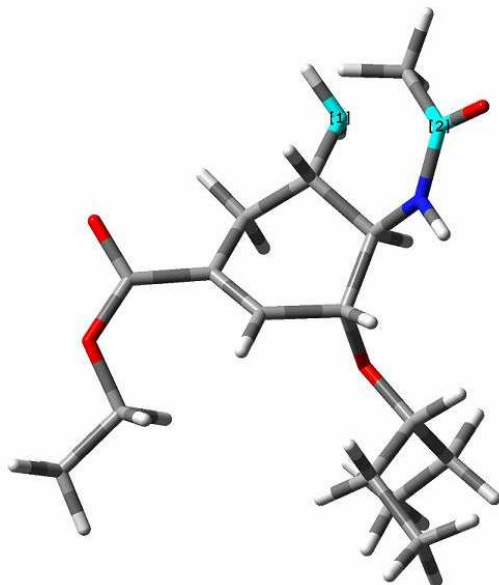
Previously, there has been performed a study on the degradation pathways for OTV (46), where Oliyai *et. al.* stated that the degradation can follow the pathways of N,N-acyl migration and/or ester hydrolysis as described in Figure 19 below.



**Figure 19: The decomposition pathways for OTV. Reprinted from (46).**

The N,N acyl migration is unlikely to occur due to the trans position of the two nitrogen atoms involved. As shown in Figure 20 on page 41, there is great distance between the amine nitrogen (the turquoise atom marked [1]) and the carbon it is supposed to react with (the turquoise atom marked [2]). This distance was measured to be 2.95 Å, which is a long distance for a reaction to occur. The angles and distances of the bonds in the structure was optimized in Chem Draw 3D 10.0, and copied to Gauss View 3.07, where the measurement of the distance was performed.

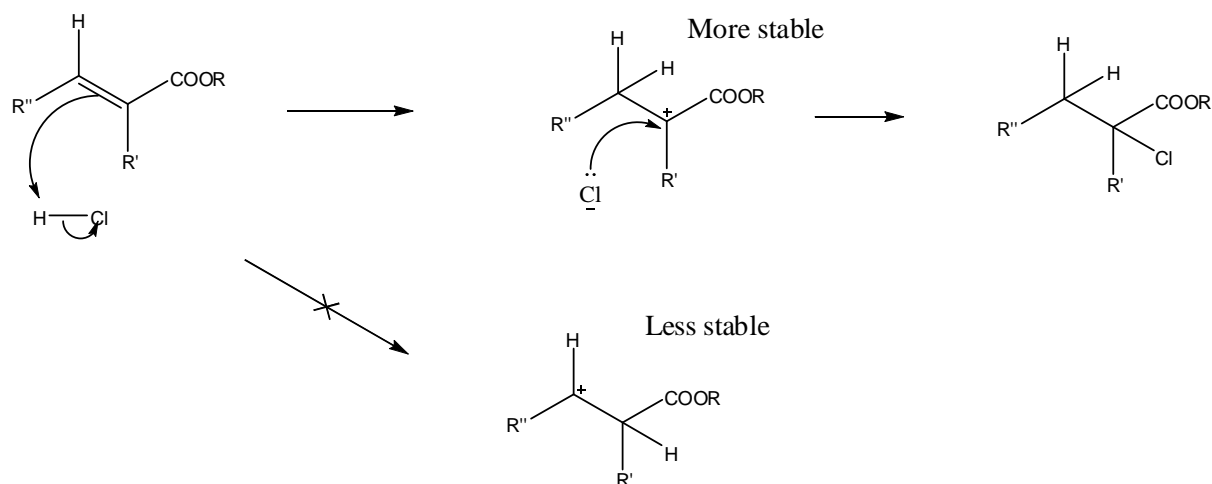




**Figure 20:** Amide and amine groups are located in a trans position to each other. Carbon atoms are marked grey, hydrogens are white, oxygens are red, amide nitrogen is blue, and the amine nitrogen and the carbon atom whom it is supposed to react with is colored turquoise.

For the electrons at the amine nitrogen to attack the acyl carbon, there has to be great movement in the structure, which requires energy. This energy can be provided as heat, as was the case for Oliyai *et. al.* (46). They performed their experiments at 70°C, while the reaction in this work was allowed to proceed in room temperature. It is therefore not likely that N,N acyl migration has occurred.

Although N,N acyl migration is doubtful to happen, addition of HCl to the double bond might occur. As shown in Figure 21 below, HCl is added in such a manner that the halide binds to the carbon with most carbon substituents. This is due to the ability to stabilize the carbocation on the carbon that is not protonated. The positive charge on the less substituted carbon is less stable and therefore not formed. This is called the Markovnikov's rule (47).

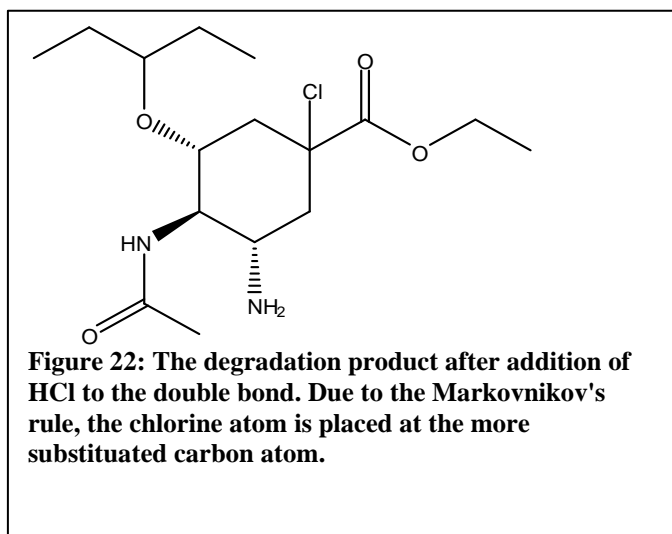


**Figure 21:** Demonstration of the Markovnikov's rule. The less substituted carbocation is less stable, and hence not formed. Reprinted from (47). It is also possible for the electrons from water to attack the carbocation, resulting in an alcohol instead of the chlorine as shown in the figure.

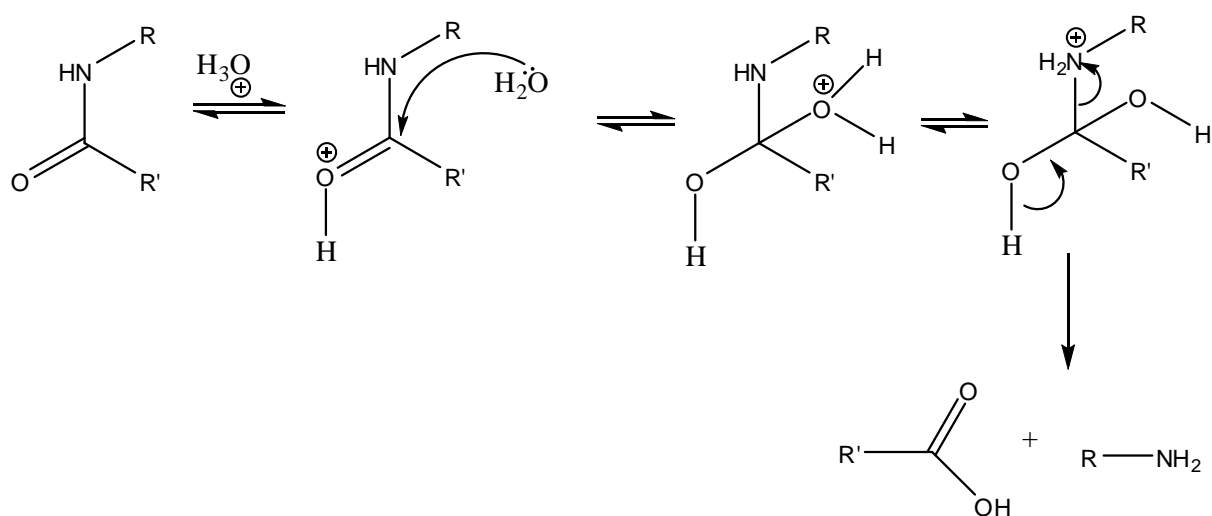
In the case of degradation due to addition of HCl to the double bond, the degradation product will be hard to detect utilizing an ultraviolet (UV) detector. Eliminating the conjugated

double bond from the structure gives the compound significantly decreased molar absorptivity. There are chromophores left in the structure (the C=O double bonds), however, the molar absorptivity of C=C double bonds conjugated with C=O are about 1.000 times higher due to an electronic transition with low probability of occurrence (47).

The problem with “invisible” substances could be overcome by using LC-MS, however, such an instrument was not available when these experiments were carried out.

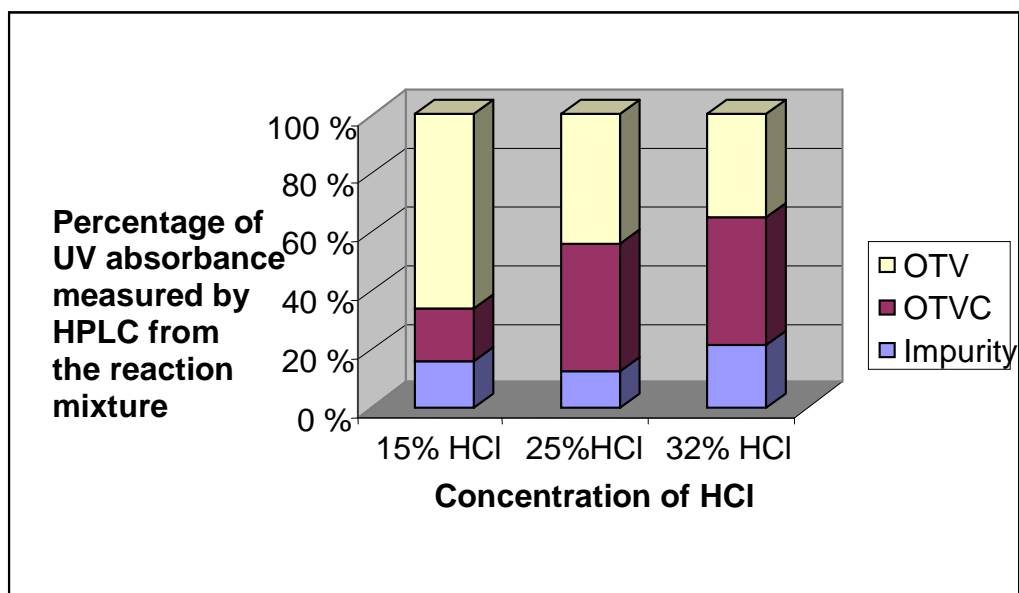


Hydrolysis of the amide catalysed by H<sub>2</sub>O and acid might also occur. Unlike amines, the C-N bond can easily be split because of the adjacent C=O bond (43). The reaction mechanism is outlined in Figure 23 below. The resulting products are an acid and an amine. On the contrary to the addition over the double bond, the decomposition product of the amide hydrolysis is visible in UV. The amides contribution for the molecule to be a chromophore is negligible as mentioned above.



**Figure 23: The reaction mechanism for acid catalysed amide hydrolysis. Reprinted from (48).**

In this work, it was performed experiments where the acid catalyzed hydrolysis were done with three concentrations of hydrochloric acid; 15 %, 25 % and 32 %. As seen in Figure 24 below the formation of OTVC in 15 % HCl is lower than in 25 % HCl. In 32 % HCl the formation of the impurity has increased, but OTVC is at the same level as in 25 % HCl.



**Figure 24:** The effect of HCl concentration on hydrolysis of OTV. The UV absorbance was measured at 230 nm with the method method 1.

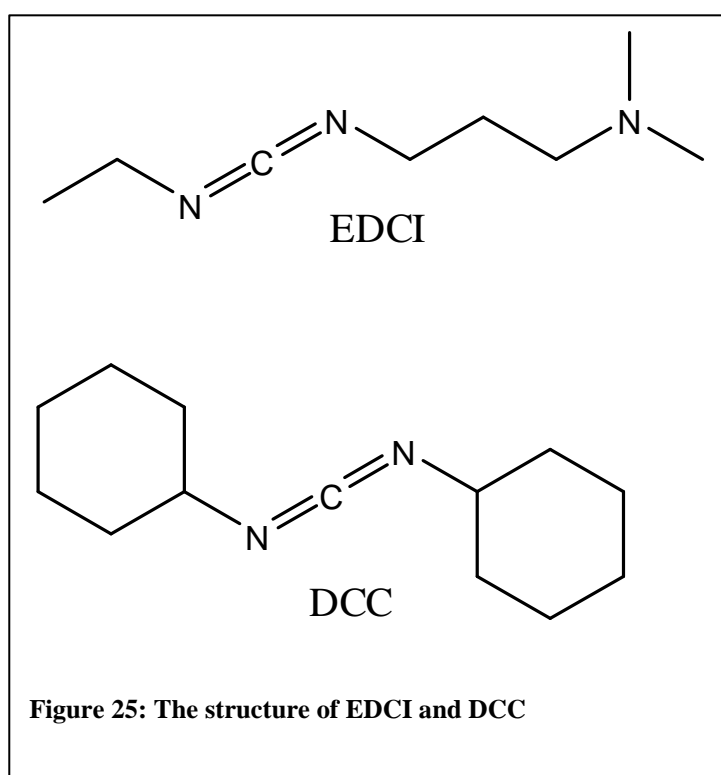
However, it is important to note that in this experiment the separation and analysis were done by high performance liquid chromatography (HPLC) with an ultraviolet (UV) detector. One of the proposed decomposing products is hardly visible in the UV and is therefore not detected. These results show only the relative concentrations of OTV, OTVC and impurity. However, since the yield of OTVC corresponded to the relative UV absorbances in the chromatogram, it was concluded that there are no “invisible” impurities are negligible.

## 7.2 Synthesis of protected ligands

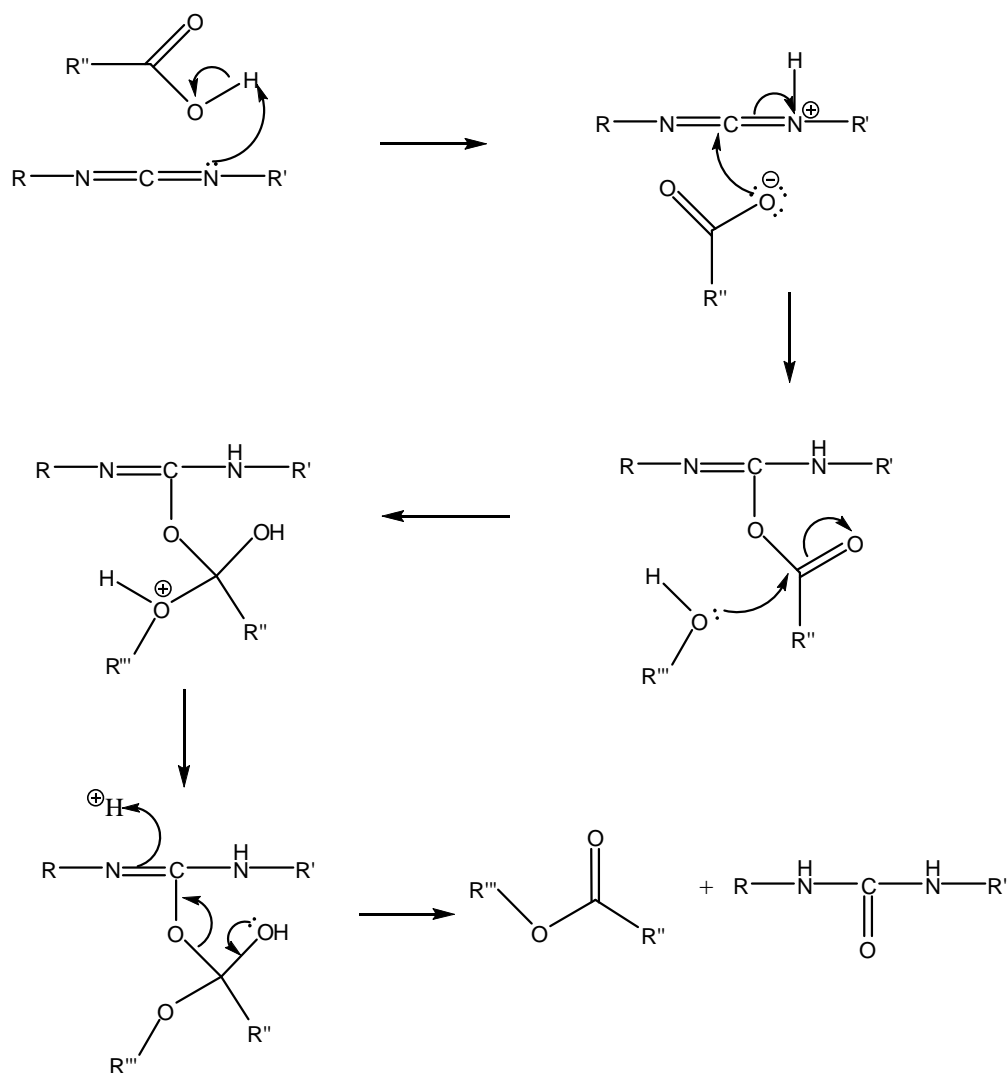
Ligands with acid and alcohol moiety were treated with excess of TCE in the presence of p-toluene sulphonic acid dissolved in toluene and refluxed in a dean stark trap. TCE was present in excess to minimize the risk for polymerization of the ligand. This resulted in a TCE ester that works as protection for the acid.

### 7.2.1 The carbodiimide coupling reagents

In this thesis, two of the carbodiimide coupling reagents have been used, namely EDCI and DCC. The differences between them are the R groups on each of the nitrogen atoms. However, both utilize the same reaction mechanism. EDCI is water soluble and can easily be removed by extraction. DCC is lipophilic and must be removed by flash chromatography or preparative HPLC. Or, if the reaction is performed in e.g. dichloromethane (DCM) or tetrahydrofuran (THF) dicyclohexyl urea can be filtrated off from the reaction mixture.



Both coupling reagents are activating carboxylic acids in order to react easily with a nucleophile. The optimum pH for EDCI has been described in an article to be 7.5 (49). At this pH, one of the nitrogen atoms are protonated. This protonation is essential for the activation to take place. The electrons of the acid (nucleophile) that is to be activated, attack the carbodiimide carbon (electrophile). The electron withdrawal effect of the carbodiimide renders the carbon of the acid more electrophilic. In this way, it is easier for the nucleophile to attack the carbonyl carbon. The nucleophile can be e.g. alcohol or amine, producing ester and amide respectively. The mechanism for the reaction with an alcohol is outlined in Figure 26 on page 45.



**Figure 26: The mechanism of the carbodiimide coupling reagents. As described in this figure, the nucleophile is an alcohol, giving an ester product. However, if the nucleophile is an amine, the product is an amide.**

### 7.2.2 Synthesis of 2,2,2.trichloroethyl 2-(4-(hydroxymethyl)phenyl)propanoate

It was tried to replace the bromine with a hydroxyl group at the commercially available precursor, and then protect the acid group with TCE. This is a new compound that has not been described in the literature before.

Tracing the reaction has shown that 20 minutes should be sufficient reaction time.

Neutralization was done with HCl, but has also been done with formic acid in other attempts of the synthesis. In other attempts of this synthesis, purification was tried with extraction from aqueous HCOOH saturated with NaCl and Na<sub>2</sub>SO<sub>4</sub> to DCM. However this was not successful.

$^{13}\text{C}$  nuclear magnetic resonance (NMR) shows two peaks that refer to carbonyl carbons. The highest carbonyl peak, 179.1 ppm, might correlate to an acid or ester. However, the lowest carbonyl peak, 162.9 ppm, seem to correlate to  $\text{ArCOOC}$  (50). Methanol ( $\text{MeOH}$ ) was used in the synthesis and evaporated. However, it is possible that the residue contained traces of  $\text{MeOH}$ , which may have been a part of an oxidation reaction, producing a methanoic ester.  $^1\text{H}$  NMR does not show any peaks near the expected singlet at about 4.61 ppm (predicted by Chem Draw Ultra 10.0) that should have correlated to the two protons  $\alpha$  to the OH and Ar. This also indicates that there has been an oxidation.

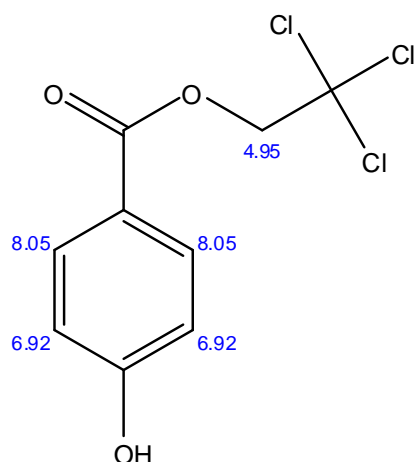
The  $^1\text{H}$  NMR showed a multiplet at 7.09 ppm. This indicates that the protons are aromatic, however, there should be four of them. The integral of 7 indicates that there are some aromatic impurities. These might be impurities of p-toluene sulphonic acid in almost one equivalent. Some of the signals from  $^{13}\text{C}$  NMR might be due to this impurity.

### 7.2.3 Synthesis of 2,2,2-trichloroethyl 4-hydroxybenzoate

The extraction was done to remove residues of p-toluene sulphonic acid. To purify the compound, the residue was dissolved in THF and mixed with RP-18, the column material in HPLC. Evaporating the THF resulted in adsorption to the particles of the material. Purification was performed by applying the column material to a pre-column on preparative HPLC. This purification method was chosen due to the large amount of residue to purify (8.88 g)

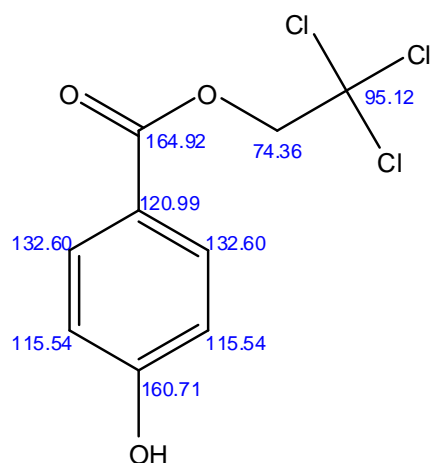
As the esterification reaction proceeds, water is produced. Since water has higher density than toluene, it is trapped in the dean stark trap. A figure of this equipment is shown in Figure 34 on page 63. A water free system is an advantage in order to avoid nucleophilic attacks of water instead of TCE.

From the  $^1\text{H}$  NMR, the signals were a singlet at 4.95 ppm that refers to the two protons at the TCE; a doublet at 6.92 ppm, with a coupling constant at 8.85 hertz (Hz), that refers to the two protons ortho to the hydroxyl group; a doublet at 8.05, with a coupling constant at 8.85 Hz, that refers to the two protons meta to the hydroxyl group. The proton at the hydroxyl group does not show in the spectrum due to deprotonation by the deuterated solvent. The shift values in ppm are shown by their respective atoms in Figure 27 on page 47.



**Figure 27: The observed <sup>1</sup>H NMR shifts values presented by their respective atoms**

From the <sup>13</sup>C NMR, the signals were at 74.36 ppm that refer to the non chlorinated carbon of the TCE moiety; 95.12 that refer to the chlorinated carbon at the TCE moiety; 115.54 ppm with integral 2 that refers to the two carbons ortho to the hydroxyl group; 120.99 ppm that refer to the carbon atom para to the hydroxyl group; 132.60 ppm with the integral 2 that refers to the two carbons meta to the hydroxyl carbon; 160.71 ppm that refer to the carbon with the hydroxyl group; 164.92 ppm that refer to the carbonyl carbon in the ester group. The shift values in ppm are shown with their respective atoms in Figure 28 below.



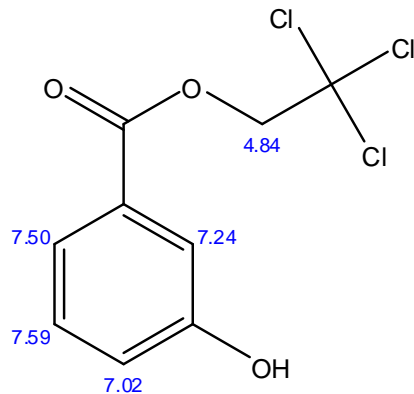
**Figure 28: The observed <sup>13</sup>C NMR shifts values presented by their respective atoms**

The mass spectrum showed three peaks at 266.9 m/z (100%), 268.9 m/z (95.6%), 270.9 m/z (8.8%) respectively. This is a typical isotope pattern from chlorine due to that about one third of all naturally occurring chlorine carry two extra neutrons in the nucleus.

### 7.2.4 Results of the synthesis of 2,2,2-trichloroethyl 3-hydroxybenzoate

This is a new substance that has not been described in the literature before. The esterification was done in a dean stark trap in order to keep the reaction conditions dry.

From the  $^1\text{H}$  NMR, the signals were a singlet at 4.84 ppm with integral of 2, which refers to the two aliphatic protons of the TCE moiety; double double doublet at 7.02 ppm with the coupling constants at 0.9 Hz, 2.6 Hz and 8.2 Hz which refer to the aromatic proton para to the ester group; triplet at 7.24 ppm with the coupling constant at 8.0 Hz, which refer to the aromatic proton ortho to both the hydroxyl and ester group; a double doublet at 7.50 ppm with the coupling constants 1.6 Hz and 2.4 Hz, which refer to the proton in para position to the hydroxyl group but ortho to the ester group; double triplet at 7.59 ppm with the coupling constants 1.2 Hz and 7.9 Hz, which refer to the proton in meta position to both the hydroxyl and ester group. The proton at the hydroxyl group does not show in the spectrum due to deprotonation by the deuterated solvent.



**Figure 29: The observed  $^1\text{H}$  NMR shifts values presented by their respective atoms**

From the  $^{13}\text{C}$  NMR, the signals were at 74.58 ppm which refer to the non chlorinated carbon at the TCE moiety; 94.83 ppm which refer to the chlorinated atom at the TCE moiety; 116.60 ppm which refer to the carbon ortho to the ester and hydroxyl group; 121.26 ppm which refer to the carbon para to the ester group; 122.49 ppm refer to the carbon para to the hydroxyl group; 129.87 ppm which refer to the carbon meta to both the hydroxyl and ester group; 130.03 ppm which refer to the aromatic carbon attached to the ester group; 155.88 ppm which refer to the aromatic hydroxyl carbon; 165.10 ppm which refer to the aliphatic ester carbon. The shift values in ppm are shown with their respective atoms in Figure 30 on page 49.



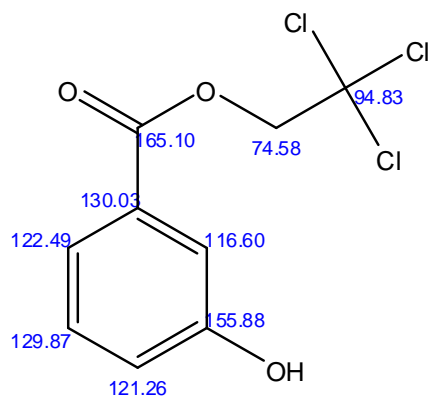


Figure 30: The observed <sup>13</sup>C NMR shifts values presented by their respective atoms

### 7.2.5 Attempted synthesis of 2,2,2-trichloroethyl 16-hydroxyhexadecanoate

The NMR results indicated that dimerization or polymerization had occurred. The peak with the highest ppm value 173.94 ppm, in the <sup>13</sup>C NMR spectrum indicates that the main product is an ester (51). The singlet peak at 4.67 ppm in the <sup>1</sup>H NMR refers to the two protons of the TCE moiety, the integral of these two protons was very low, and was set to 2 because it should account for 2 protons, compared to the larger peaks, this indicates that the desired TCE-ester might be present but in a very low quantity.

The triplet at 3.56 refers to the two protons α to the hydroxyl group. Since the two peaks mentioned refer to the same number of protons, it indicates that the rest of the 16-hydroxy hexadecanoate molecules have esterified. The shift value of the hydrogen α to the alcohol shall be about 3.50 ppm, while shift value to the corresponding hydrogen in an ester is about 4.13 ppm (prediction done with Chem Draw Ultra 10.0). At 3.98 ppm, there is a triplet that accounts for 22 protons, which refers to the α protons on the alcohol side of the ester on the dimer. The triplet at 2.39 refers to the protons on the carbon α to the carbonyl side of the ester of the product.

With help from Marius Standal, an LCMS of the compound was performed, which showed a lot of peaks showed. Among these, was the expected peak at 402 m/z, however, without the characteristic chlorine isotope pattern. This indicates that the desired product is not present. When looking at all of the ions detected by the MS, a peculiar pattern of masses was discovered. Nine ions were detected with 44 m/z separating each of them. The signals

increased in intensity from 452.5 m/z to 584.6 and 628.6, and decreased with the higher m/z values. The values were: 452.5 m/z (15 %), 496.5 m/z (32 %), 540.5 m/z (73 %), 584.6 m/z (100 %), 628.6 m/z (100 %), 672.6 m/z (66 %), 716.6 m/z (32 %), 760.6 m/z (12 %), 804.6 m/z (4 %). No rational explanation for this pattern was found.

Primary aliphatic alcohols are better nucleophiles than the alcohol of TCE due to the inductive effect of chlorine. Therefore the electron pair on the OH is less localized. For the same reason, an aromatic alcohol is a poor nucleophile related to a primary aliphatic alcohol. This is probably the reason to why this reaction went well with the aromatic compounds, and not with the aliphatic alcohol.

Due to limited supplies of the main reactant, this reaction was performed in a smaller scale than the other reactions with TCE. However, the volume of the solvent was not reduced equivalently. The result was a more dilute reaction mixture than the other TCE coupling reactions. It is a fact that 16-OH hexadecanoic acid can react with it self. In addition, a dilute reaction mixture favors the intramolecular lactonization. However, when the alcohol and acid is separated by such a long carbon chain, it is unlikely that an intramolecular lactonization can occur. This usually happen when the lactone ring become a 5 or 6 membered ring.

Although the synthesis was done with an excess of TCE, this reaction did not proceed as expected.

#### **7.2.6 Attempt of coupling 2,2,2.trichloroethyl 4-hydroxybenzoate with $\gamma$ -amino butyric acid**

This was meant as a model synthesis, where  $\gamma$ -amino butyric acid (GABA) replaced OTV since the molecule contains both an amino group and a carboxylic acid group.

At first, the reactants were tried to dissolve in dimethylformamide (DMF) only, with subsequent adding of small quantities of concentrated sulfuric acid in order to dissolve the reactants. A total of 182 mg sulfuric acid was needed. Ultrasound bath was also needed for the dissolution to take place. After the purification, recrystallization from DCM was tried in order to produce a powder. However, this was not successful.

Dimethylaminopyridine (DMAP) was added as a catalyst for the reaction to take place. EDCI was added when the reaction was stirred on ice bath. The EDCI is a coupling reagent that activates the acid. The carbonyl carbon is electron deficient and is attacked by a nucleophile. There are two nucleophiles in the reaction, the phenol and the amine. Since the amine was protonated due to the acidified solution it was a poor nucleophile and would therefore not be able to attack the activated acid. However, the same can be said about the phenol. For the phenol to be a good nucleophile, it needs a higher pH in order to be deprotonated.

The isolated compound had the same retention time in HPLC as the reactant (TCE protected p-hydroxybenzoate). However, the main peak of the mass spectrum showed a compound weighing 174.1 g/mol, not correlating with any of the compounds involved. This peak lacks the typical chlorine isotope fragmentation and can therefore not contain any chlorine atoms. The peak at 147.0 is about 33 % of the size of the peak at 145. This indicates that the 145 peak contains a chlorine atom. It has not been possible to correlate any of the masses to a plausible product/side product of the reaction. The desired product was not observed in the mass spectrum.

There are mainly two reasons for the failure of this experiment. Both are related to the pH. In order to dissolve the reactants, the solution was acidified. First, the low pH made the phenol unable to do a nucleophilic attack on the acid. Secondly, the EDCI was protonated due to the excess of acid. This rendered the acid unable to do the nucleophilic attack on the activated EDCI, and hence might not activated (See the detailed mechanism in Figure 26 on page 45).

### 7.2.7 Attempted protection of oseltamivir carboxylate with isonicotinyl

The isonicotinylloxycarbonyl has previously been used by Veber *et.al.* as protection group for amines. The isonicotinyl p-nitrophenyl carbonate used in this synthesis was obtained from a colleague that previously had synthesized the compound based on the article of Veber *et.al.* (52).  $^1\text{H}$  NMR of this compound was in accordance with the structure of the compound.

$\text{CuCl}_2$  was added to the solution as a catalyst. It is thought that  $\text{Cu}^{2+}$  complexes with the oxygen at the carbonyl, rendering the carbonyl carbon more electropositive and therefore

easier to attack by a nucleophile. After the reaction, H<sub>2</sub>S is bubbled through the solution in order to precipitate the Cu from the solution as CuS (52).

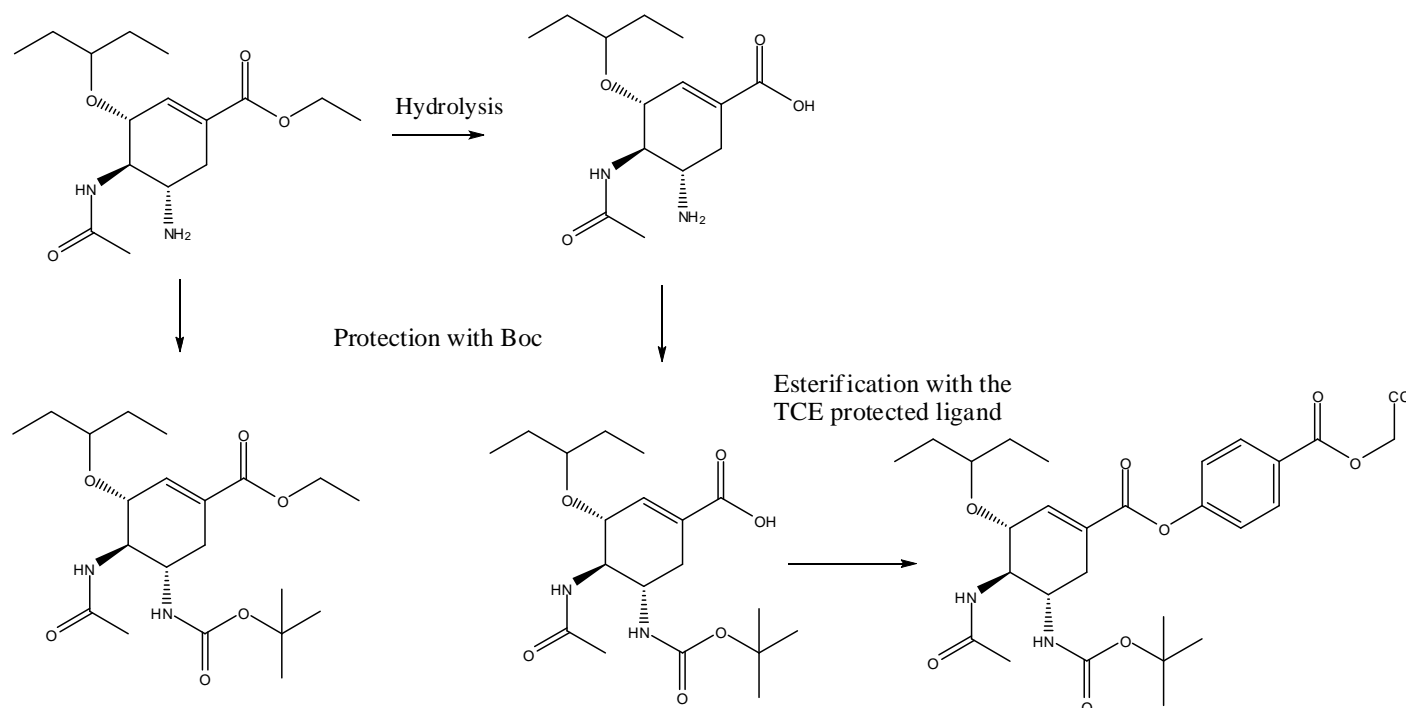
The main peak from the mass spectrum, 275.1, indicates that the isolated compound was p-nitrophenyl carbonate [M+H]<sup>+</sup>. The <sup>1</sup>H NMR and <sup>13</sup>C NMR, however, was not equally unambiguous. The singlet at 5.38 ppm could correlate to the aliphatic protons and the doublets at 6.89 ppm, 7.47 ppm, 8.11 ppm and 8.31 ppm could correlate to the aromatic protons of p-nitrophenyl carbonate if all signals were equally big. However integrals of the signals at 6.89 ppm and 8.11 ppm are half the size of the other signals. The <sup>13</sup>C NMR showed nine signals, which do not correlate to the p-nitrophenyl carbonate that produce only eight signals.

The NMRs and MS were not consistent since MS indicated that one of the reagents was isolated, while NMR indicated otherwise. Any way, the product was not observed neither in MS nor NMR. It was therefore decided not to proceed with isonicotinyl as the protection group for the amine.

#### **7.2.8 Synthesis of 2,2,2-trichloroethyl 4-((3R,4R,5S)-4-acetamido-5-(tert-butoxycarbonylamino)-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoate**

Since the end product could be separated from the other side products in the synthesis it was thought to be unnecessary to perform any purification between each reaction step. An HPLC of the final residue showed three of the compounds showed in Figure 31 on page 53. When Boc<sub>2</sub>O was added to the solution, the amines on both OTV and OTVC were protected. The esterification was able only to take place in the molecules that were hydrolyzed in the first step. The Boc protection seemed to be complete due to the fact that OTV and OTVC were not seen in the chromatogram after the reaction with Boc<sub>2</sub>O.

The carboxylic acid of OTVC-Boc was activated by DCC. The DCC reaction was allowed to stir for 2 days.



**Figure 31: The five compounds of the synthesis. Only the lower left compound was desired.**

The purification was done by preparative HPLC using the eluent  $\text{H}_2\text{O}:\text{MeOH}$  (20:80) with 10 milli molar (mM)  $\text{HCOOH}$ . With this eluent, the other substances were eluted first. After 30 minutes, the compound was eluted with 100% MeOH. Since the compound is very lipophilic, it interacts well with the  $\text{C}_{18}$  material of the column. 100% MeOH was therefore needed in order to elute the compound.

The mass spectrum showed that the title compound was isolated. The main signal was 657.2 m/z, which correspond to the  $[\text{M}+\text{Na}]^+$ , with the typical pattern for a molecule with three chlorine atoms.  $^1\text{H}$  NMR indicates that this compound is present. Most of the shift values from the spectrum correspond to the desired molecule as shown in Figure 32 on page 54. One of the protons does not show due to moisture in the sample. None of the signals from the compound lacks in the spectrum, however, there are other signals that indicates that impurities are present. The integral of a characteristic aliphatic proton on the ring that did not seem to overlap with other signals was set to one. This gave the signals from the aromatic protons an integral of about 5 that should have been 2, and the aliphatic protons on the ether had integrals of 21 that should be 6, and 25 that should be 4. As seen, the integrals do not correspond well to each other in the way they should. This indicates that unwanted side products may have formed. Since the NMR showed that the compound was not completely dry, and some side products may have formed, a yield of 28.2 % may be overestimated.

Although the reaction yielded 131 mg, just a small portion thereof was used for the NMR analyses. The rest was used in the subsequent experiments. The concentration was therefore too thin for the  $^{13}\text{C}$  NMR to give a reliable spectrum.

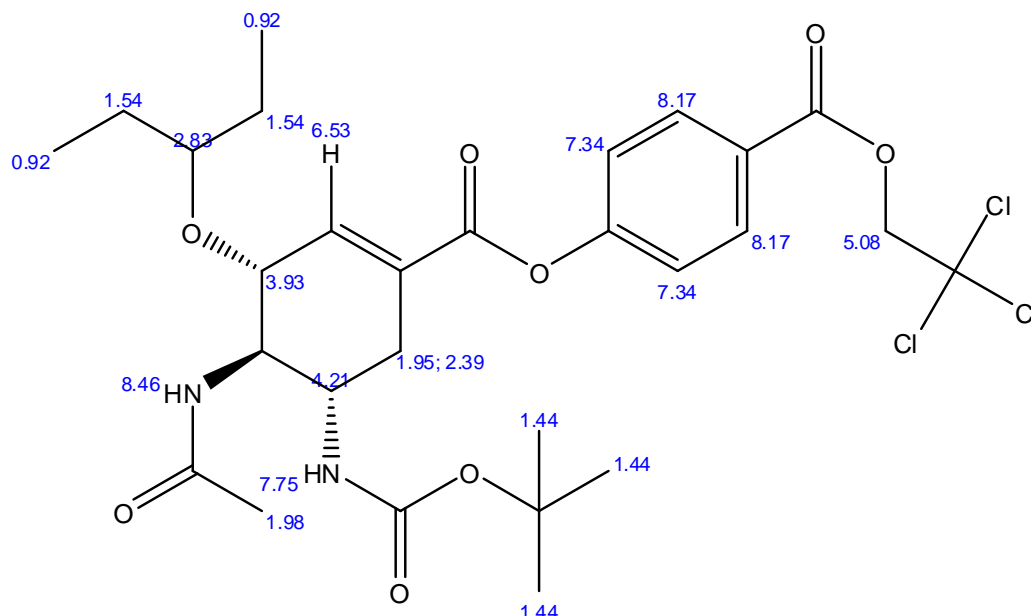


Figure 32: The  $^1\text{H}$  NMR shift values of the title compound showed by their respective atoms.

### 7.2.9 Attempted synthesis of 4-((3R,4R,5S)-4-acetamido-5-amino-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoic acid

The two protection groups present in the structure, are Boc and TCE. Boc is deprotected under acidic conditions and TCE with acid in the presence of Zn in a catalytic amount. Although it has been described a procedure to deprotect TCE with 90 % acetic acid, it was chosen to utilize concentrated acetic acid in order to reduce the risk of acid catalyzed hydrolysis of the ester bond in the molecule.

The multiplets from 1.22 ppm to 1.92 ppm in  $^1\text{H}$  NMR indicate that the isolate contained a large amount of aliphatic impurities. These signals in addition to the triple triplet at 3.52 ppm seem to originate from dicyclohexyl urea (DCU) which is a side product after the usage of DCC. This was used in the previous reaction and the reaction mixture was purified by preparative HPLC. It is possible that DCU has coeluted with the product of this reaction. DCU is hardly visible in UV (at 254 nm) and can therefore not be discovered at that stage of synthesis.

This theory was confirmed by the mass spectrum. The mass of DCU is 224 g/mol and the mass of 225.2 m/z is therefore  $[M+1]^+$  of DCU. Traces of the title compound were not seen.

The  $^1\text{H}$  NMR of the starting material showed a singlet at 1.44 ppm that referred to the protons of Boc. This signal is absent in this  $^1\text{H}$  NMR, which indicates that the Boc group has been deprotected. However, the aromatic protons and the aliphatic protons at the TCE group still appear, indicating that this group has not been deprotected. The odd thing is that these protons appear with integrals that are twice as large as the rest of the protons in OTVC molecule should be. This could mean that half of the TCE protected molecules has hydrolyzed in the ester bond between OTVC and the ligand.

Five of the signals in  $^{13}\text{C}$  NMR may originate from DCU. These are the signals at 24.1 ppm, 24.8 ppm, 32.8 ppm, 47.6 ppm, 157.8 ppm. This means that there is only one peak left in the carbonyl area (164.9 ppm), where there should be three signals. Signals from both OTVC and the ligand has been seen, however, all of the carbonyl signals was not seen. This is most likely due to a thin sample.

Even though the  $^1\text{H}$  NMR did not lack any of the expected signals, MS and  $^{13}\text{C}$  NMR has indicates that the desired compound is not present. However, the compound may be present in small quantities.

### 7.3 Crystallographic studies

At first, the crystals of oseltamivir phosphate (OTVP) and OTVC were tried to be grown by dissolving them in various solvents and allowing solvents to evaporate from open vials over night as described in Table 4 below. In most of the glasses this resulted in an amorphous glassy formation, while in others, crystals had grown. However, the crystals had grown in such a rapid manner that they grew into each other, looking almost like glass in the microscope. This knowledge lead to the conclusion that crystals for x-ray might be grown if the growing process are allowed to slow down.

**Table 4: Experiments with crystal growing in open vials.**

#### **Crystal growing**

Experiment	Compound	Solvent
1	20 mg OTVC	100 µl H <sub>2</sub> O
2	20 mg OTVC	200 µl MeOH
3	20 mg OTVC	300 µl EtOH + 300 µl EtOH
4	20 mg OTVP	100 µl H <sub>2</sub> O
5	20 mg OTVP	200 µl MeOH
6	20 mg OTVP	600 µl EtOH + 600 µl EtOH

Crystals of the compounds, OTV and OTVC, were then tried to be grown in a slow manner, allowing a liquid precipitant slowly to diffuse into the chamber where the compounds are dissolved in water. The precipitant must be volatile, miscible with water, and the compound must be insoluble in the liquid. Slow but surely, the solubility of the compound will decrease as more of the liquid dissolves in the water. Finally, a grain will precipitate and the compound will precipitate onto this corn, resulting in a crystal growing slowly.

The slow crystal growing experiments were carried out as described in section 9.8 on page 75.



**Table 5: The experiments carried out for growing crystals.**

Exp. No.	Compound	Precipitant	Result
1	OTVC	ACN	No crystals
2		Ethanol	No crystals
3		Isopropanol	No crystals
4		Acetone	Crystal growth
5	OTVP	ACN	Crystal growth
6		Ethanol	No crystals
7		Isopropanol	No crystals
8		Acetone	No crystals

As seen in Table 5 above, several of the most commonly used precipitants such as ethanol and isopropanol resulted in no precipitation, and hence no crystal growth. Acetone resulted in crystal growth for OTVC, but not for OTV, and vice versa for ACN. To our great surprise, neither ethanol nor isopropanol resulted in any crystal growth in either of the substances although both substances are insoluble in both precipitants.

Although crystals from both OTV and OTVC were grown, they were not the appropriate size for achieving an x-ray from them. When crystals grow, they grow in the energetically most favorable manner. That is, if it takes more energy to grow in the x and y direction than it takes to grow in the z direction, the resulting crystals will grow long and thin. This was the case for both OTV and OTVC. The crystals were several  $\mu\text{m}$  long but were measured to be about 10  $\mu\text{m}$  thick. To be able to produce any reliably x-ray structures, the crystals must have a thickness of at least 30  $\mu\text{m}$ .

In order to grow thicker crystals it is possible to alter the growing conditions, like using another precipitation agent, adding different salts to the solution or altering the temperature. However, if salts are added to the solution, they might grow into the crystals and alter the conformation of the molecule studied. On the other hand, three dimensional conformation of the molecule might not be the same in a crystal and in the solution. This means that the conformation obtained from the crystal might not be the same as is present in the body.

## 8 Concluding remarks

A strategy for synthesis was worked out that included the hydrolysis of OTV, protection of amide on OTVC with Boc, protection of the ligands acid with TCE, esterification of the ligand with OTVC, and finally deprotection of the protecting groups. It was first tried to protect the amine with isonicotinyl, but since this was unsuccessful, the amine was protected with Boc instead.

There has been synthesized a series of protected ligands for OTVC, and one of them was successfully esterified with the Boc protected OTVC. However, due to lack of time, it was not possible to find a proper way to deprotect the two protecting groups. Further research is needed in this area in order to obtain these products. Since no prodrugs were obtained, no protein binding studies were performed either.

A few attempts have been made of crystallizing OTV and OTVC, since this has not been done before. After two weeks, there were obtained crystals from both compounds. However, the crystals were too thin to produce any reliable results from the x-ray.

## 9 EXPERIMENTAL

### 9.1 Materials

#### 9.1.1 Reagents

Chemicals	Supplier	Quality
16-hydroxy hexadecanoic acid	Aldrich	>98%
2,2,2-trichloroethanol	Aldrich	>99%
2-[4-(bromo-methyl)phenyl]propanoic acid	Aldrich	>99%
4-amino butyric acid	Koch-Light Laboratories	Pure CHR
Di cyclo carbodiimide	Fluka	~99%
Di-methyl amino pyridine	Fluka	>98%
Di-tert-butyl dicarbonate	Janssen Chimica	>97%
EDCI	Fluka	>98%
hydrochloric acid min 32%	Riedel-de Haën	>32%
isopropanol	A/S Vinmonopolet	
Magnesium sulphate anhydrous	Fluka	>98%
m-hydroxy benzoic acid	The British Drug Houses LTD	>99%
potassium hydroxide	Fluka	>98%
potassium iodine	Fluka	>98%
p-toluenesulphonic acid	Merck	pro analysi
RP-18	Amicon	
sodium hydrogen carbonate	Aldrich	>99.5%
sodium sulphate	Fluka	>99.0%
Sulphuric acid	Merc	95-97%
Zn pulverized	Unknown	

#### 9.1.2 Solvents

absolutt alkohol prima	Arcus	
acetic acid	NMD	Concentrated
dichloromethane	Fluka	>99,9%
ethyl acetate	Fluka	>99.5%
formic acid	Merck	98-100%
methanol	Sigma-Aldrich	>99.9%
tetra hydrofurane	Kebolab	99.5%
toluene	Riedel-de Haën	>99.9%
	Elga PureLab Maxima	
	HPLC (ionexchanged,	
	HPLC-grade water)	
water		

### 9.1.3 Solvents for NMR

Solvents for NMR	Supplier	Quality
Chloroform-d	Aldrich	99.8%
Deuterium oxide	Aldrich	100 %
Methanol d <sub>6</sub>	Aldrich	>99.8%

## 9.2 ESI-MS

All ESI-MS were recorded on Waters/Micromass Q-TOF2 instrument with either positive or negative ionization detection mode.

## 9.3 HPLC analysis

### 9.3.1 Apparatus

#### 9.3.1.1 Technical details

Autosampler:	HP G1313A
Pump:	HP Agilent 1100 series binary pump
Column:	C18 250 x 2 mm, 5 μ particle size, Luna, Phenomenex
Detector:	HP G1315A Diode Array Detector
Data acquisition and handling:	HP Chemstation version.A.06.03 [509]

#### 9.3.1.2 Experimental parameters

Column temperature:	Ambient
Injection volume:	5 μl
Flow rate:	0.5 ml/min
Mobile phase:	Gradient or isocratic
Chromatogram run time	Specified in each method
Detection wavelength:	UV, 317 nm and 250 nm

### 9.3.2 Mobile phase for method 1

Time (min)	% ACN	% Formic acid 10 mM, pH 3	Flow (ml/min)
0	20	80	0.5
8	80	20	0.5
12	80	20	0.5
14	20	80	0.5

## 9.4 Preparative HPLC

### 9.4.1 Apparatus

#### 9.4.1.1 Technical details

Injector:	Manual
Pump:	Modified HP 1050
Column:	250 x 20 mm packed with LiChroprep®RP-18, 25-40 µm particles
Detector:	Labomatic Labcord-200 UV spectrometer

#### 9.4.1.2 Experimental parameters

Column temperature:	Ambient
Injection volume:	5 – 10 ml
Flow rate:	5 - 10 ml/min
Mobile phase:	Isocratic
Detection wavelength:	UV, 254 or 210 nm, $\pm$ 8 nm

### 9.4.2 Mobile phase

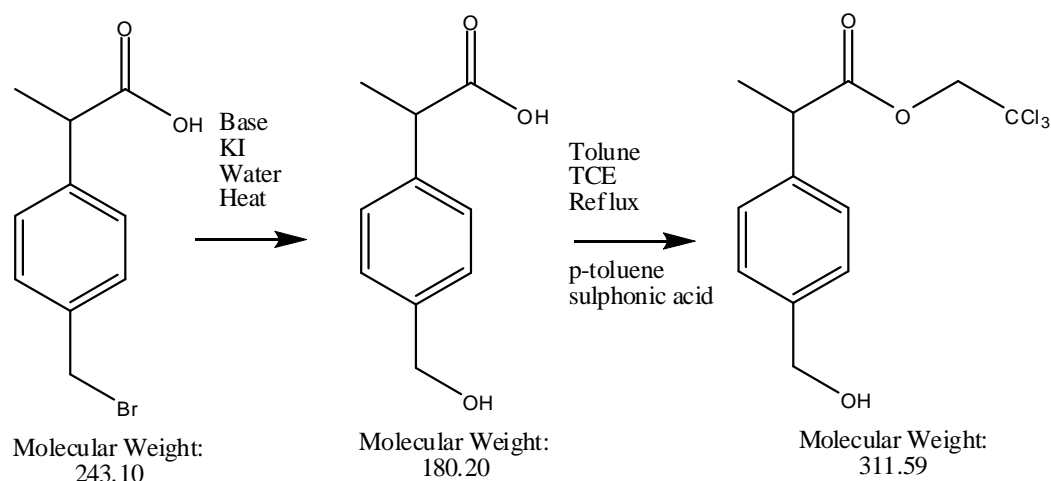
The optimum isocratic mobile phases were obtained using several volume ratios of ACN and formic acid solution at pH 3 on analytical HPLC. The isocratic eluent that gave satisfactory separation with retention times of about 6 minutes was chosen as the mobile phase.

**9.5 NMR analysis**

All  $^1\text{H}$  and  $^{13}\text{C}$  NMR analyses were recorded on a Bruker Avance DPX 300 instrument at 300MHz and 75 MHz respectively. All experiments were performed at 25°C with deuterated solvents.

## 9.6 Syntheses of the ligands

### 9.6.1 Synthesis of 2,2,2-trichloroethyl 2-(4-(hydroxymethyl)phenyl)propanoate



**Figure 33** Synthesis scheme for 2,2,2-trichloroethyl 2-(4-(hydroxymethyl)phenyl)propanoate

2-(4-(Bromo methyl)phenyl)propanoic acid (0.504 g, 2.07 mmol), potassium iodine (one grain) and potassium hydroxide (1.125 g 20.0 mmol, 56.11 g/mol) were dissolved in water (10 ml), and was allowed to reflux for 20 minutes. HCl solution (12 ml 5%) was added to neutralize to pH 5. The water was evaporated under vacuum and the residue was dissolved in MeOH producing a precipitate which was filtered. MeOH was evaporated, yielding a dry powder (592 mg). Toluene (50 ml) was added together with TCE (0.932 g, 6.24 mmol, 149.40 g/mol) and p-toluenesulphonic acid (1.771 g, 10.3 mmol, 172.20 g/mol). This was allowed to reflux for 2 hours, and stirring at room temperature over night. The solution was evaporated producing a dry powder (2,207 g) which was dissolved in DCM (40 ml) and washed with 3 portions of H<sub>2</sub>O (40 ml). DCM was evaporated for 20 minutes at 80°C to remove remaining residues of toluene. The residue was dissolved in H<sub>2</sub>O:ACN (20:80) and purified on preparative HPLC with H<sub>2</sub>O:ACN (30:70) + 10 mM HCOOH as the eluent. A compound was isolated (214 mg)

<sup>1</sup>H-NMR (300 MHz):

δ 1.40 (d, 3H, J=7.2Hz), 1.45 (s, 1H), 2.16 (s, 1H), 2.23 (s, 2H), 2.80 (s, 3H), 2.87 (s, 3H), 3.61 (q, 1H, J=7.0Hz), 3.83 (s, 1H), 3.88 (s, 1H), 7.09 (m, 7H), 7.95 (s, 1H)

$^{13}\text{C}$  NMR (75 MHz):

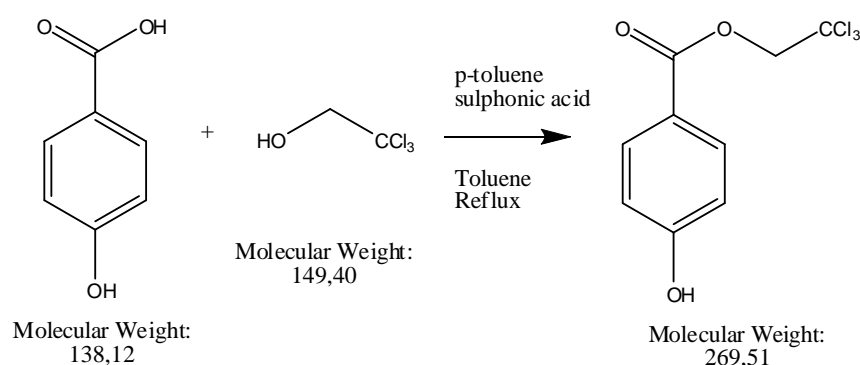
$\delta$  18.17, 19.64, 20.96, 27.37, 31.53, 36.59,  
 38.96, 41.08, 44.89, 125.94, 126.41, 127.54,  
 127.60, 128.76, 128.88, 128.99, 129.09,  
 129.88, 130.22, 135.51, 136.53, 137.67,  
 137.71, 137.84, 138.73, 139.33, 140.40,  
 162.92, 179.36

There were some overlapping signals.

MS ( $\text{ES}^+$ ; TOF):

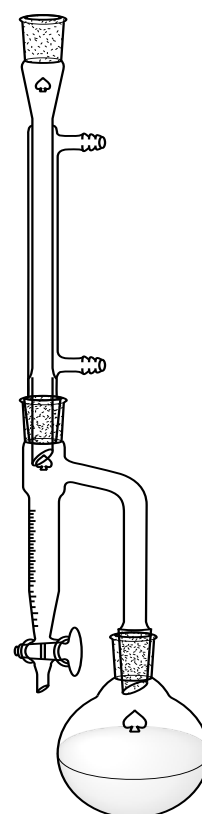
Not done.

### 9.6.2 Synthesis of 2,2,2-trichloroethyl 4-hydroxybenzoate



**Figure 35 Synthesis of 2,2,2-trichloroethyl 4-hydroxybenzoate**

P-hydroxy benzoic acid (4.048 g, 29.31 mmol), TCE (12.279 g, 82.1 mmol) and p-toluenesulphonic acid (18.890 g, 109.6 mmol, 172.20 g/mol) were dissolved in toluene (100 ml). The solution was refluxed in a dean-stark trap (Figure 34) for 15 minutes. The solution was evaporated and water (100 ml) and DCM (100 ml) was added to the residue and the phases were separated and the aqueous phase was extracted twice more with DCM (100 ml). The DCM was evaporated, which resulted in a dry grey white powder (8.88 g). The powder was dissolved in THF. A white powder consisting of RP-18, three times the volume of the pre-column was added to the solution, and THF was evaporated. The grey white powder adsorbed to the RP-18 was put in a pre-column and was



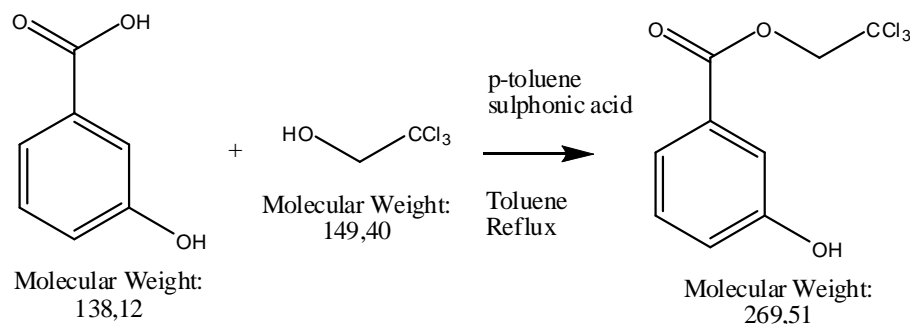
**Figure 34 The equipment used for Dean Stark Trap**



purified on a preparative HPLC, with an eluent consisting of H<sub>2</sub>O:ACN (40:60) + 10 mM HCOOH. This was done three times, due to the volume of RP-18. The eluent was evaporated, and the residue was dissolved in ACN, resulting in a product with the consistence and colour of honey. This was dissolved in DCM and evaporated, yielding a white dry powder (5.072 g, 18.81 mmol, 64.2 % yield, 99.3 % purity determined by HPLC). The retention time in HPLC was 9.8 min. using method 1.

<sup>1</sup> H NMR (300 MHz, CDCl <sub>3</sub> ):	<p>δ 4.95 (2H, s), 6.92 (2H, d, <i>J</i> = 8,85 Hz), 8.05 (2H, d, <i>J</i> = 8,85 Hz)</p> <p>The hydroxyl proton does not show</p>
<sup>13</sup> C NMR (75 MHz, CDCl <sub>3</sub> ):	<p>74.36 (1 C, s), 95.12 (1 C, s), 115.54 (2 C, s), 120.99 (1 C, s), 132.60 (2 C, s), 160.71 (1 C, s), 164.92 (1 C, s)</p>
MS (ES <sup>+</sup> ; TOF):	<p>266.9 (100%), 268.9 (95.6%), 270.9 (8.8%)</p> <p>[M + H]<sup>+</sup>, [M + H + 2]<sup>+</sup>, [M + H + 4]<sup>+</sup></p> <p>The percentages were measured with a ruler.</p>

### 9.6.3 Synthesis of 2,2,2-trichloroethyl 3-hydroxybenzoate

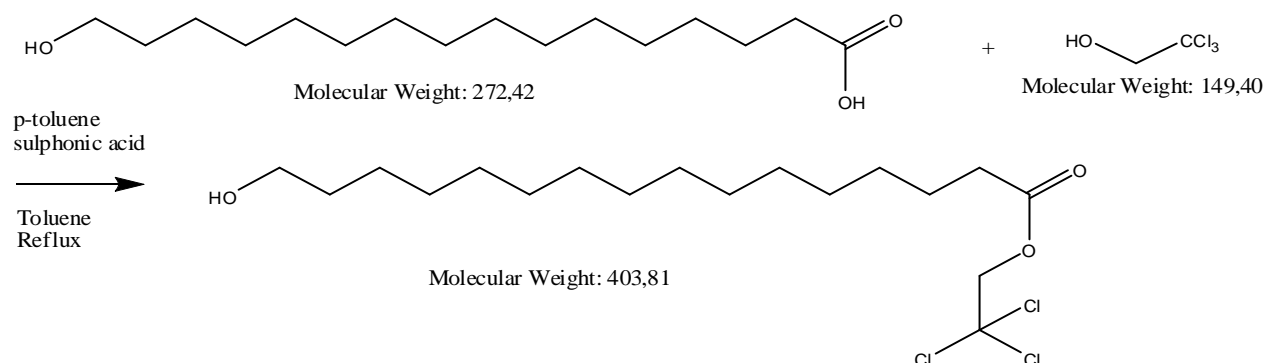


**Figure 36** Synthesis of 2,2,2-trichloroethyl 3-hydroxybenzoate

m-hydroxybenzoic acid (0.537 g, 3.88 mmol), TCE (1.524 g, 10.20 mmol) and p-toluenesulfonic acid (1.725 g, 10.02 mmol, 172.20 g/mol) were dissolved in toluene (130 ml) and refluxed at in a Dean Stark trap for 90 minutes. The solution was evaporated, and the residue was dissolved in DCM and washed with water (3\*30 ml). The organic phase was evaporated and dissolved in a H<sub>2</sub>O:ACN (20:80 4 ml). This was applied on a preparative HPLC for purification, using the eluent H<sub>2</sub>O:ACN with HCOOH (30:70). The title compound was isolated, resulting in a white powder (0.153 g, 0.568 mmol, 14.6 % yield)

<sup>1</sup> H-NMR (300 MHz, CDCl <sub>3</sub> )	<p>δ 4.84 (s, 2H), 7.02 (ddd, 1H, J=0.9Hz, J=2.6Hz, J=8.2Hz), 7.24 (t, 1H, J=8.0Hz), 7.50 (dd, 1H, J=1.6Hz, J=2.4Hz), 7.59 (dt, 1H, J=1.2Hz, J=7.9 Hz)</p>
<sup>13</sup> C-NMR (75 MHz, CDCl <sub>3</sub> )	<p>δ 74.58 (s, 1C), 94.83 (s, 1C), 116.60 (s, 1C), 121.26 (s, 1C), 122.49 (s, 1C), 129.87 (s, 1C), 130.03 (s, 1C), 155.88 (s, 1C), 165.10 (s, 1C)</p>
MS (ES <sup>+</sup> ; TOF):	<p>266.9 (100%), 268.9 (95.9%), 270.9 (10.1%) [M + H], [M + H + 2], [M + H + 4] The percentages were measured with a ruler.</p>

#### 9.6.4 Attempted synthesis of 2,2,2-trichloroethyl 16-hydroxyhexadecanoate



**Figure 37** Synthesis of 2,2,2-trichloroethyl 16-hydroxyhexadecanoate

16-hydroxyhexadecanoic acid (98 mg, 0.36 mmol), p-toluenesulfonic acid (141 mg, 0.818 mmol, 172.20 g/mol) and TCE (153 mg, 1.02 mmol) were dissolved in toluene (50 ml) and refluxed in a dean stark trap for 90 minutes. The solution was evaporated and the residue was dissolved in ACN (100 ml) and extracted hexane (3\*100 ml). The hexane phase was filtered, which yielded a white dry powder (50 mg)

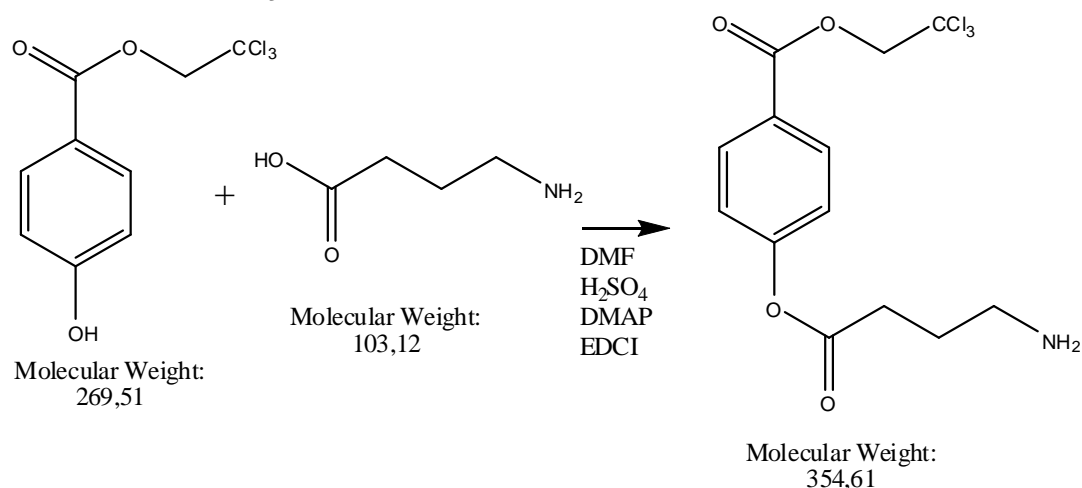
$^1\text{H}$  NMR:  $\delta$  1.19 (m, 265H), 1.54 (m, 45H), 2.22 (m, 22H), 2.33 (m, 3H), 2.39 (m, 3H), 3.56 (t, 2H,  $J=6.6\text{Hz}$ ), 3.98 (t, 22H), 4.67 (s, 2H)

$^{13}\text{C}$  NMR:  $\delta$  28.31, 28.57, 29.09, 29.19, 29.20, 29.51, 29.57, 34.34, 40.72, 64.33, 77.20, 173.94  
There were some overlapping signals

MS: See discussion in section 7.2.5 on page 49.

## 9.7 Coupling of the ligands with a model substance

### 9.7.1 Attempt of coupling 2,2,2-trichloroethyl 4-hydroxybenzoate with $\gamma$ -amino butyric acid

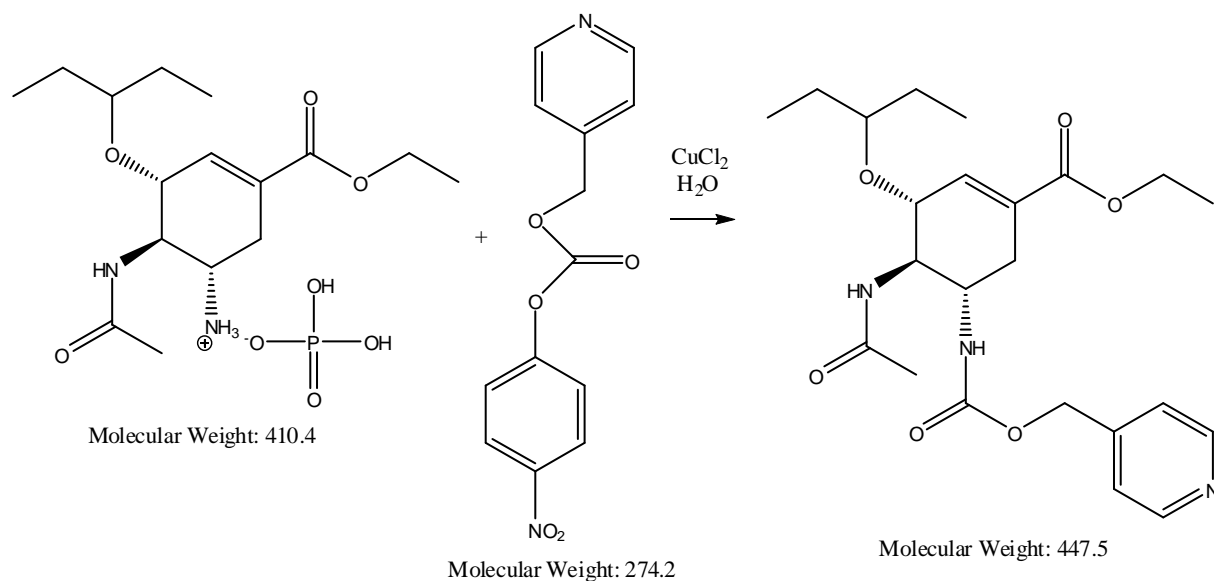


**Figure 38 Attempt to synthesize 2,2,2-trichloroethyl 4-(4-aminobutanoyloxy)benzoate**

DMF (10 ml) was acidified with concentrated sulphuric acid (182 mg) in a 50 ml round bottomed flask. 2,2,2-trichloroethyl 4-hydroxybenzoate (251 mg, 0.931 mmol) and 4-aminobutanoic acid (107 mg, 1.04 mmol) were added. The flask was stirred in ultrasound bath till the entire solid was dissolved. (DMAP) (One grain) was added and dissolved by stirring at ultrasound bath. The solution was put on an ice bath with a magnetic stirring. N-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI) (362 mg, 1.89 mmol, 191.7 g/mol) was added and dissolved using ultrasound bath. 5 minutes after the EDCI was added and dissolved, the reaction mixture was removed from the ice bath and was stirred for 30 minutes. The HPLC showed a slight shortening of retention time using the method 1 (8.8 minutes). An array of isocratic runs were ran on the HPLC to find the optimum eluent for the preparative HPLC. After evaporation of the reaction mixture, the residue was purified on preparative HPLC using the eluent  $H_2O:ACN$  (30:70) + 10 mM  $HCOOH$ . Fraction 4 and 5 was combined before evaporation. This resulted in a thick liquid (267 mg).

MS ( $ES^+$ ; TOF): 145.0 (7.5 %), 147 (2.5 %) 174.1 (100 %), 313.2 (5.6 %)

### 9.7.2 Attempted protection of oseltamivir carboxylate with isonicotinyl



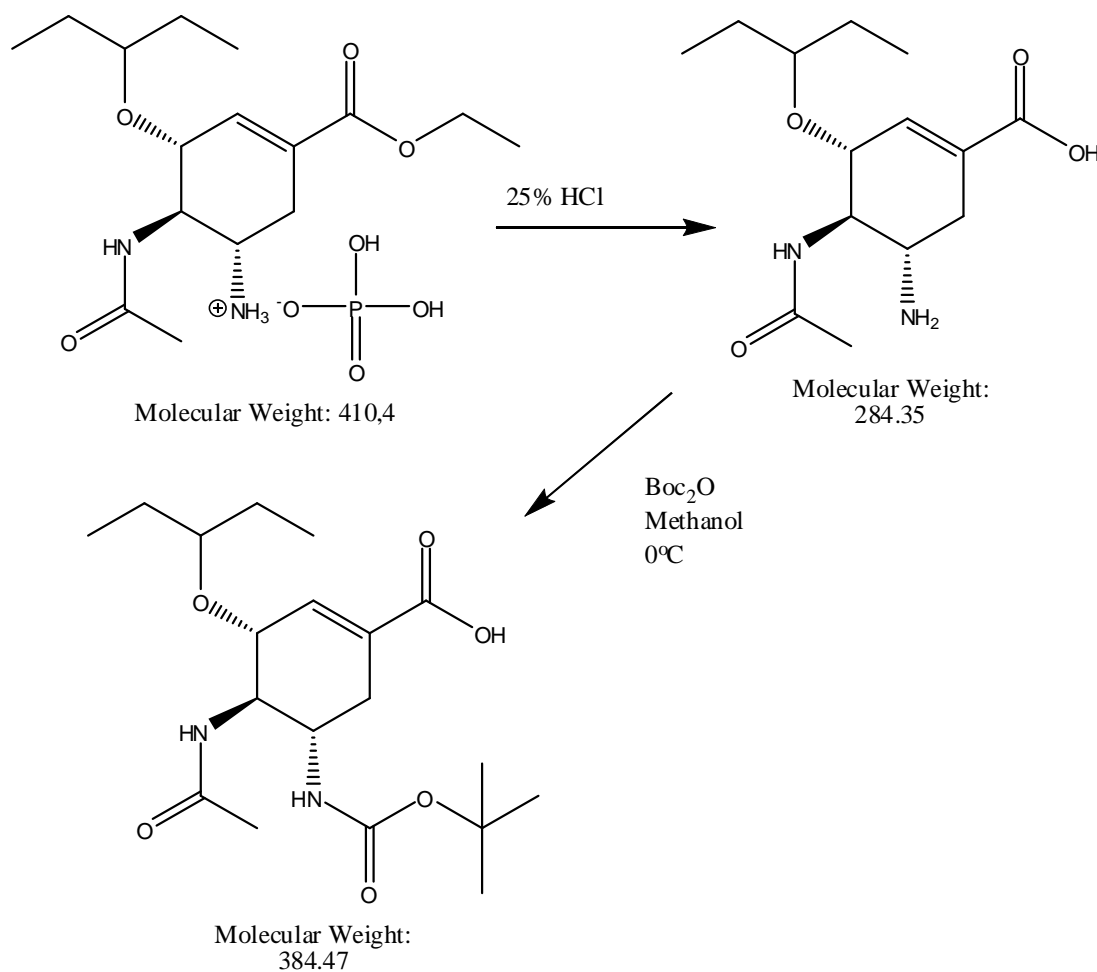
A solution of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (37 mg, 0.25 mmol, 147 g/mol) in water (0.65 ml) was added to a solution of OTV (103 mg, 0.251 mmol, 410.4 g/mol) in water (0.65 ml). The pH was adjusted to 9.5 with NaOH (25% aqueous solution). Isonicotinyl p-nitrophenylcarbonate (91 mg, 33 mmol) was added and the solution was stirred at ultrasound bath. The solution changed color from green to blue. The solution was stirred over night at room temperature on a magnetic stirrer. One drop of acetic acid neutralized the solution to pH 7. The solution was filtered and washed with water. The solid was dissolved in ACN and was bubbled with  $\text{H}_2\text{S}$  gas for 30 minutes. The gas was obtained from a system where FeS was allowed to react with HCl (5 % aqueous solution). The solid was filtered off and the solvent was evaporated.

$^1\text{H}$  NMR (300 MHz):  $\delta$  5.38 (s, 2H), 6.89 (d, 1H,  $J=9.1\text{Hz}$ ), 7.47 (d, 2H,  $J=9.1\text{Hz}$ ), 8.11 (d, 1H,  $J=9.1\text{Hz}$ ), 8.31 (d, 2H,  $J=9.1\text{Hz}$ )

$^{13}\text{C}$  NMR (75 MHz):  $\delta$  69.04, 122.57, 125.96, 126.71, 141.11, 146.32, 153.11, 156.20, 164.48  
There were some overlapping signals

MS ( $\text{ES}^+$ ; TOF): 157.1 (64 %), 219.1 (23 %), 275.1 (100 %), 413.3 (3 %), 453.8, (5 %), 485.8 (6 %)

### 9.7.3 Speed of oseltamivir hydrolysis

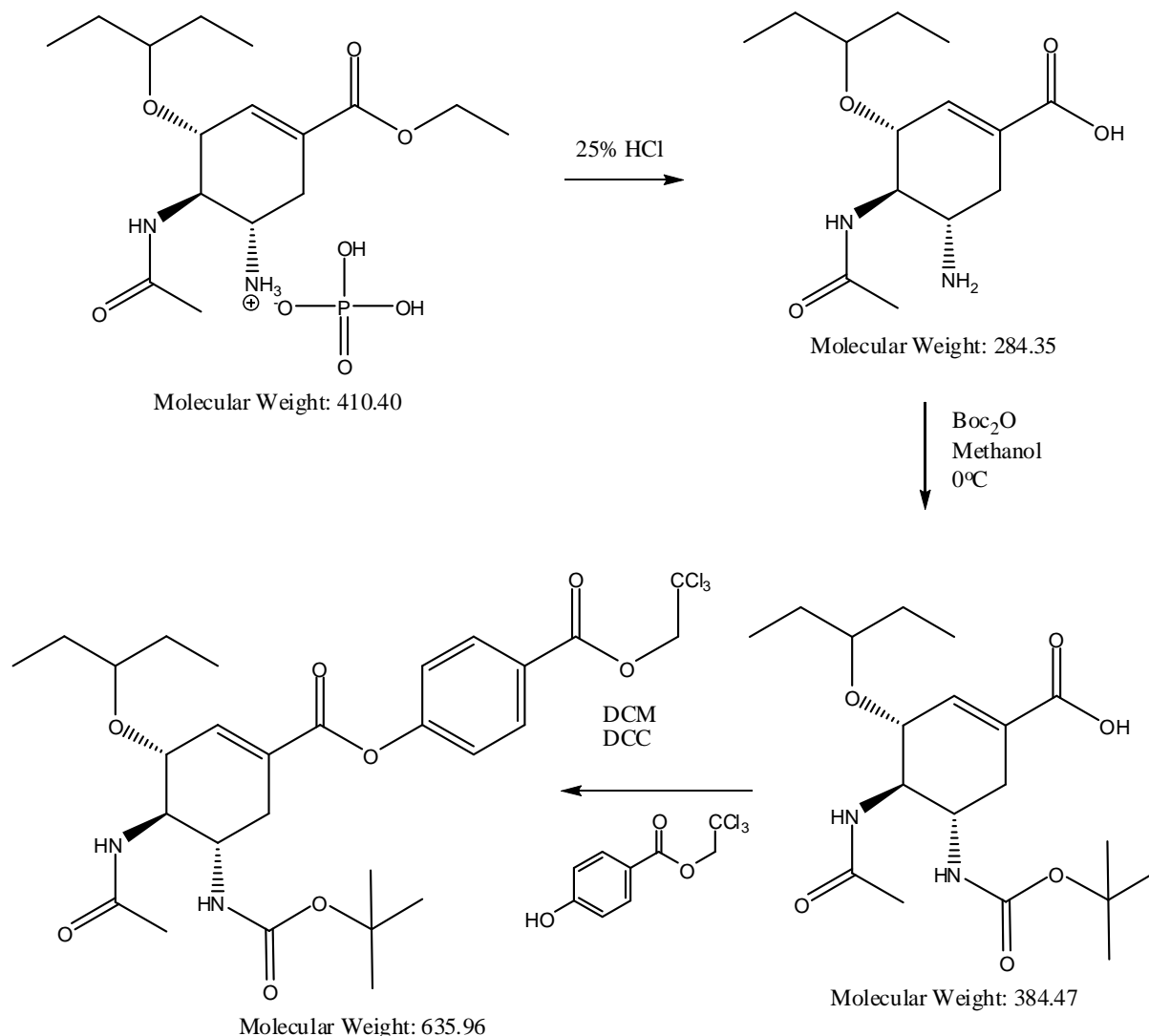


**Figure 39** Synthesis scheme for the hydrolysis of OTV.

OTVP (499 mg, 1.22 mmol) was dissolved in HCl (1.567 g, 25 % solution) and was stirred on a magnetic stirrer for 1 day. A part of the acidic OTV solution (415 mg) was neutralized with  $\text{NaHCO}_3$  to pH 7. A few drops of water were added to dissolve the base. The solution was added dropwise to a suspension of  $\text{MgSO}_4$  (11.5 g) in MeOH (100 ml) on a magnetic stirrer and was allowed to stir for 15 minutes. The suspension was filtered and the volume was reduced by evaporation to about 20 ml. A white powder precipitated during the evaporation. This was filtrated off before next reaction. The solution was put on ice bath and  $\text{Boc}_2\text{O}$  (59 mg 0.27 mmol) was dissolved in MeOH (2 ml) and added dropwise on magnetic stirrer. After 5 minutes the reaction was allowed to stir in room temperature for 2 hours.

The next day, another part of the acidic OTV solution (409 mg) was neutralized with  $\text{NaHCO}_3$  to pH 7. The solution was dried, filtered, evaporated and was allowed to react with  $\text{Boc}_2\text{O}$  (56 mg, 0.26 mmol, 218.2 g/mol) as the day before. HPLC analyses were run of the reaction mixtures from both days.

### 9.7.4 Synthesis of 2,2,2-trichloroethyl 4-((3R,4R,5S)-4-acetamido-5-(tert-butoxycarbonylamino)-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoate



**Figure 40** Synthesis scheme for 2,2,2-trichloroethyl 4-((3R,4R,5S)-4-acetamido-5-(tert-butoxycarbonylamino)-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoate

Oseltamivir phosphate (298 mg, 0.726 mmol) was dissolved in HCl (1.016 g 25%) and was stirred on a magnetic stirrer for 1 day. The solution was neutralized with NaHCO<sub>3</sub> to pH7. A few drops of water were added to dissolve the base. It takes about 590 mg NaHCO<sub>3</sub> to neutralize 1 g 25 % HCl solution. A suspension of Na<sub>2</sub>SO<sub>4</sub> (10 g) in MeOH (100 ml) was prepared. The neutralized OTV solution was added dropwise to the MeOH suspension on a magnetic stirrer. The suspension was filtered on a glass filter with suction and washed with MeOH. The solution was evaporated at 25°C till the remaining volume was about 20 ml. During evaporation more white powder seemed to precipitate from the solution. The

suspension was filtered on a paper filter in a glass funnel. Boc<sub>2</sub>O (244 mg, 1.12 mmol, 218.25 g/mol) was dissolved in MeOH (2 ml) and added dropwise to the solution on ice bath with magnetic stirring. After 5 minutes on ice bath, the reaction was allowed to stir at room temperature over night. The solution was evaporated at 25°C and the residue was dissolved in DCM (20 ml). 2,2,2-trichloroethyl 4-hydroxybenzoate (202 mg, 0.750 mmol) was added and dissolved using ultrasound bath. The solution was allowed to cool down on ice bath. N,N-dicyclohexylcarbodiimide (DCC) (151 mg, 0.731 mmol, 206.33 g/mol) was dissolved in DCM (2 ml) and added dropwise to the solution on ice bath with magnetic stirring. The reaction was allowed to stir for 2 days. The solution had a milky look, and was still milky after a filtration. The filtrate was evaporated and dissolved in ACN. The solution was still milky until the addition of water. A clear precipitate appeared which was filtered off and removed. The filtrate was evaporated at 25°C and dissolved in MeOH (2 ml) and purified on preparative HPLC using the eluent H<sub>2</sub>O:MeOH (20:80) with 10 mM HCOOH. After 30 minutes, the eluent was switched to pure MeOH which eluted a substance with retention time 12.8 minutes using method method 1. The title compound was isolated (131 mg, 0.205 mmol, 90.1 % purity measured by HPLC, 28.2 % yield)

<sup>1</sup>H-NMR (300 MHz, MeOH d<sub>6</sub>)

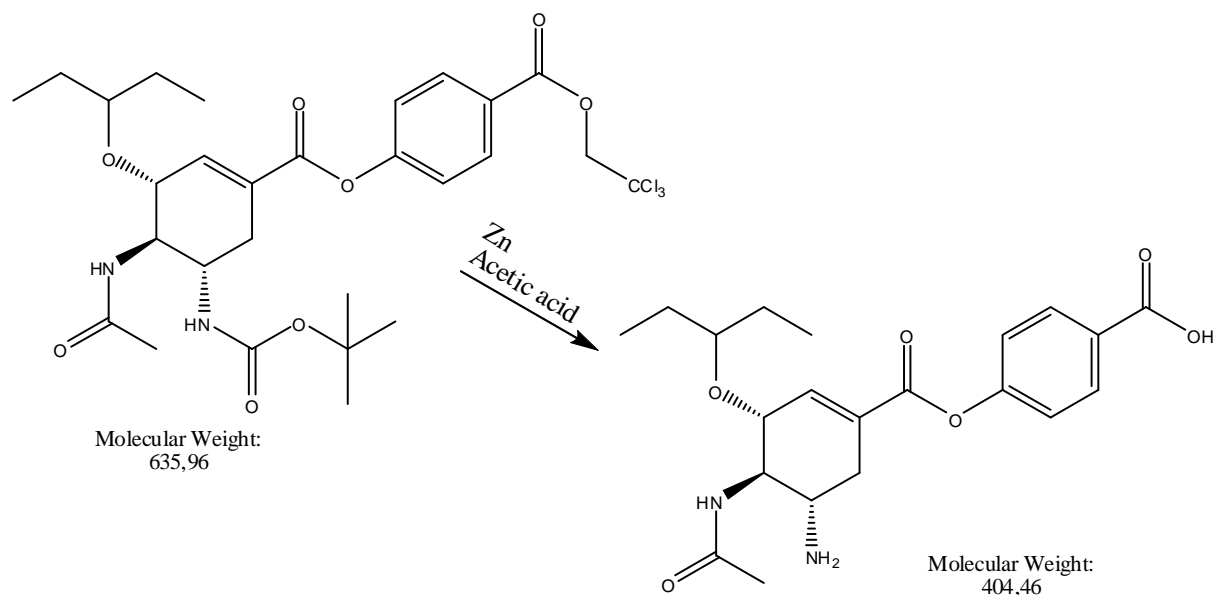
δ 0.92 (td, 21H, J=7.3Hz, J=10.6Hz), 1.29 (s, 14H), 1.44 (s, 25H), 1.54 (m, 13H), 1.95 (m, 1.4H), 1.98 (s, 6.8H), 2.39 (m, 0.91H), 2.83 (dd, 1H, J=5.1Hz, J=17.6Hz), 3.35 (s, 5.5H), 3.46 (m, 1.8H), 3.82 (m, 0.5H), 3.93 (dd, 0.9H, J=8.4Hz, J=11.1Hz), 4.21 (m, 1.62H), 5.08 (s, 7H), 6.53 (d, 0.9H), 7.03 (m, 1.9H), 7.34 (d, 4.8H, J=8.9 Hz), 8.11 (s, 5.4H), 8.17 (d, 5.4H, J=8,9 HZ),

One proton does not show due to moisture in the sample.

MS (ES<sup>+</sup>; TOF): 657.2 (100%), 659.2 (95 %), 661.2 (11 %)  
[M+Na]<sup>+</sup>, [M(<sup>37</sup>Cl)+Na]<sup>+</sup> and [M(<sup>37</sup>Cl<sub>2</sub>)+Na]<sup>+</sup>  
Respectively



### 9.7.5 Attempted synthesis of 4-((3R,4R,5S)-4-acetamido-5-amino-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoic acid



**Figure 41** Synthesis scheme for 4-((3R,4R,5S)-4-acetamido-5-amino-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoic acid

Pulverized zinc (catalytic amount) was added to anhydrous acetic acid (5 ml). 2,2,2-trichloroethyl 4-((3R,4R,5S)-4-acetamido-5-(tert-butoxycarbonylamino)-3-(pentan-3-yloxy)cyclohex-1-enecarbonyloxy)benzoate (26 mg, 0.041 mmol) was added and the solution was stirred for 1 hour. The acetic acid was evaporated to about 2 ml. This was purified on preparative HPLC using H<sub>2</sub>O:ACN (40:60) with 10 mM HCOOH as the eluent. Fractions 7 and 8 were combined and evaporated producing white solid (18 mg) with retention time 9.6 using method 1 on HPLC.

<sup>1</sup>H-NMR (300 MHz) :

δ 0.96 (m, 21H), 1.22 (m, 50H), 1.41 (m, 46H), 1.62 (m, 30H), 1.76 (m, 32H), 1.92 (m, 32H), 2.04 (m, 3H), 2.10 (s, 6H), 2.47 (ddt, 2H, J=2.8Hz, J=10.2Hz, J=16.3Hz), 2.97 (dd, 2H, J=5.5Hz, J=17.4Hz), 3.52 (tt, 21H, J=4.1Hz, J=10.7Hz), 3.99 (dd, 2H, J=8.5Hz, J=11.2Hz), 4.30 (q, 8H, J=7.1Hz), 5.03 (s, 2H), 6.93 (d, 2H, J=8.9Hz and m, 2H), 7.79 (s, 2H), 8.03 (d, 2H, J=8.9Hz), 8.14 (s, 1H)

$^{13}\text{C}$ NMR:	$\delta$ 7.8, 8.1, 12.7, 21.4, 24.1, 24.7, 24.8, 25.2, 27.8, 32.8, 47.6, 48.6, 60.4, 73.2, 73.6, 77.2, 81.9, 114.5, 126.5, 131.4, 137.2, 157.8, 164.9,
MS ( $\text{ES}^+$ ; TOF):	225.2 m/z (100 %)

## 9.8 Crystallographic studies

OTVP and OTVC (about 0.5 – 2 mg, enough to cover the bottom of the tube) was put in two respective tubes and dissolved in water (30  $\mu$ l). The tubes were capped by wrapping parafilm on the top, and punctured with a thin needle. The tube containing OTVC was lowered into a glass containing acetone (1 ml), while the tube containing OTVP was lowered into a glass with ACN (1 ml), as shown in Figure 42 below. The glasses were sealed with screw caps, and placed in a dark place in room temperature for 2 weeks.



**Figure 42:** Picture of the equipment used for crystal growth. The inner tube is capped with parafilm perforated with a needle to make a small hole. The liquid is allowed to diffuse through the small hole and dissolve in the water, precipitating the substance slowly onto a crystal.

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